Formation and Maintenance of GABAergic Synapses

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Zusammenfassung

Der grösste Teil der Inhibition im Gehirn findet an GABAergen Synapsen statt. Daher ist ihre korrekte Entwicklung elementar für eine einwandfreie Gehirnfunktion. Die Bildung von GABAergen Synapsen geschieht in mehreren Stufen: in einem ersten Schritt aggregieren prä- und postsynaptische Proteine an der richtigen Stelle. Darauf folgt die Erkennung der Bindungspartner zwischen dem Axon und dem Dendrit. Nach erfolgter Aggregation und Bindung aller prä- und postsynaptischen Elementen werden neugebildete Synapsen in einem letzten Schritt der Synapsenreifung stabilisiert oder eliminiert. Bis heute sind die Mechanismen, wie die Initiierung der Synapsenbildung abläuft, wie neugeborene Synapsen stabilisiert und aufrechterhalten werden, sowie welche Proteine und Signalwege in diese Prozesse involviert sind, noch nicht vollständig geklärt. Die Zielsetzung dieser Dissertation war es, ein besseres Verständnis dieser Mechanismen zu erhalten, mit besonderem Schwerpunkt auf Aggregation von Proteinen an der postsynaptischen Dichte. Wir wollten die wichtigsten Proteine dieser Prozesse identifizieren sowie eine Hierarchie in der Proteinaggregation und der darauffolgenden GABAergen Synapsenbildung herleiten. Der Fokus lag hauptsächlich auf dem $GABA_A$ Rezeptor sowie Gepyhrin, Neuroligin2 und Collybistin, weil diese Proteine schon in früheren Studien als potentielle Kandidaten für solche Prozesse identifiziert wurden. Diese Fragestellungen wurden mittels eines *in vitro* Ansatzes in neuronalen Primärkulturen sowie anhand zweier *in vivo* Projekten an knockout Mäusen, welchen jeweils eines der untersuchten Gene fehlt, studiert.

Das erste Projekt war der Fragestellung gewidmet, welchen Einfluss GABAerge Innervation auf frühe Stadien der Synaptogenese ausübt. Dazu wurden hippocampale Neuronen in niedriger Zellkulturdichte verwendet, welche dadurch zu Beginn der Synaptogenese keine GABAerge Innervation erfahren. In diesen Kulturen aggregieren GABAerge postsynaptische Proteine auf der gegenüberliegenden Seite von glutamatergen präsynaptischen Nervenendigungen, wo sie „fehlverknüpfte" Synapsen bilden. Synapsen wurden visualisiert durch Immunfluoreszenzfärbungen der postsynaptischen Proteine, sowie deren Lokalisation relativ zu angefärbten Markerproteinen der GABAergen oder glutamatergen präsynaptischen Nervenendigungen. Daraus ging hervor, dass die Bildung von Synapsen ein zellautonomer Prozess ist, welcher unabhängig von den präsynaptisch abgegebenen Neurotransmittern stattfindet. Um GABAerge Innervation zu induzieren, wurden EGFP-exprimierende GABAerge Neuronen einmal vor und einmal während der Synaptogenese, diesen Kulturen beigefügt. Diese Experimente zeigten, dass die Anwesenheit GABAerger präsynaptischer Nervenendigungen bei der Initialisierung der Synaptogenese die Bildung von fehlverknüpften Synapsen unterbindet. Im Gegensatz dazu werden bereits gebildete Fehlverknüpfungen bei späterer GABAerger Innervation nicht eliminiert. Dies deutet auf einen kompetitiven Mechanismus für die Interaktion mit bereits etablierten postsynaptischen Aggregaten hin. GABAerge postsynaptische Proteinaggregate, welche von einer GABAergen sowie einer glutamatergen präsynaptischen Nervenendigung gleichzeitig innerviert werden, suggerieren die

Induktion einer korrekten präsynaptischen Innervation durch bereits bestehende postsynaptischer Aggregate.

Die Frage, welche Proteine wichtig sind für die Induktion und Stabilisierung von postsynaptischen Aggregaten, wurde *in vivo* durch immunhistochemische Analyse von mutierten Mäusen untersucht. Zuerst wurden Mäuse, welche kein Collybistin exprimieren, analysiert. In der Literatur wird Collybistin als wichtiges Protein für die Aggregation von Gepyhrin vorgeschlagen. In Purkinjezellen dieser Mäuse ist Gephyrin nie aggregiert, weder während der Entwicklung noch im adulten Tier. Dennoch aggregieren Neuroligin2 und der $GABA_A$ Rezeptor auf den Dendriten an den richtigen Stellen genau gegenüber GABAerger präsynaptischer Nervenendigungen. Dies deutet darauf hin, dass weder Gephyrin noch Collybistin für die Aggregation dieser Proteine und die Bildung GABAerger Synapsen essentiell sind.

Der Einfluss der $GABA_A$ Rezeptoren auf die Bildung und Stabilität GABAerger Synapsen wurde im ventrobasalen Komplex des Thalamus von $GABA_A$ Rezeptor α1-Untereinheit-knockout Mäusen untersucht. Dieses Projekt wurde in Kollaboration mit Prof. Jeremy Lambert durchgeführt, welcher mit seiner Gruppe parallel zu unseren morphologischen Analysen, elektrophysiologische Messungen an diesen Tieren durchgeführt hat. In thalamischen Relay-Neuronen werden während der zweiten postnatalen Woche $GABA_A$ Rezeptoren, welche die α2-Untereinheit inkorporiert haben, durch α1-haltige $GABA_A$ Rezeptoren ersetzt. Diese α1-haltigen $GABA_A$ Rezeptoren vermitteln synaptische inhibitorische Transmission im ventrobasalen Komplex von erwachsenen Tieren. In neugeborenen α1-knockout Mäusen werden GABAerge Synapsen korrekt gebildet, wie aus Färbungen für prä- und postsynaptische Proteine sowie aus elektrophysiologischen Messungen hervorging. Nach der zweiten postnatalen Woche hingegen, wird die α2-Expression herunter reguliert und α1-knockout Mäuse weisen keine synaptischen $GABA_A$ Rezeptoren mehr auf. Dieser entwicklungsbasierte Verlust von $GABA_A$ Rezeptor Expression führt zum Abbau der synaptischen Gepyhrin Aggregate und die Synapsen sind elektrophysiologisch nicht mehr aktiv. Dennoch bleiben die präsynaptischen Nervenendigungen bestehen und unterscheiden sich in Anzahl und Grösse nicht von jenen in Wildtyp Mäusen. Ultrastrukturelle Analysen haben gezeigt, dass diese Nervenendigungen spezialisierte nicht-synaptische Verbindungen mit Dendriten von Relay-Neuronen eingehen, welche möglicherweise Orte der Neurotransmitterausschüttung darstellen, die für tonische Inhibition von extrasynaptisch lokalisierten Rezeptoren im ventrobasalen Komplex dieser Mäuse verantwortlich sind. Diese Resultate zeigten, dass funktionale Transmission zwar wichtig ist um Synapsen zu stabilisieren, präsynaptische Nervenendigungen aber ohne funktionale Transmission dennoch nicht degenerieren, sondern neuartige Kontakte mit den Zielzellen eingehen.

Zusammenfassend zeigen diese Resultate, dass postsynaptischen Elementen eine übergeordnete Rolle bei der Bildung und Stabilisierung von GABAergen Synapsen zugeordnet werden kann. Obwohl keines der hier untersuchten Proteine essentiell ist für die Ausbildung GABAerger Synapsen kann eine Hierarchie in ihren Interaktionen aufgestellt werden. Dabei scheint Neuroligin2 eine Schlüsselrolle in der lokalen Bestimmung der Synapsenbildung zu spielen, wohingegen postsynaptische Aggregation von Gepyhrin vom $GABA_A$ Rezeptor abhängig ist.

Summary

GABAergic synapses comprise the major sites of inhibition in the brain and their correct development is crucial for proper brain function. Formation of GABAergic synapses is a multistep process: First, pre- and postsynaptic proteins cluster at the appropriate subcellular sites, followed by partner recognition among the target axon and dendrite and finally, synapses are stabilized or eliminated in a process of synapse maturation. It is not well understood how the formation of GABAergic synapses is initiated, how nascent synapses are stabilized and maintained, and which proteins and signaling pathways are involved in these processes. The aim of this thesis was to gain further insight into these mechanisms, with a special focus on clustering of proteins of the GABAergic postsynaptic density. We aimed to identify the key players for these processes and to deduce a hierarchy in protein clustering and subsequent GABAergic synaptogenesis. In particular we focused on the $GABA_A$ receptor, gephyrin, neuroligin2 and collybistin, because these proteins have been shown previously to be likely candidates playing a role in these processes. We studied these issues in three projects using an *in vitro* approach in primary cultured hippocampal neurons as well as two *in vivo* approaches in mice, deficient for one of the proteins of interest.

The first project aimed to understand the influence of GABAergic input during early stages of synaptogenesis. Therefore, low-density hippocampal cultures were used, which do not receive GABAergic input at the onset of synaptogenesis. In these cultures, GABAergic postsynaptic proteins cluster apposed to glutamatergic terminals where they form apparent "mismatched" synapses in absence of GABAergic input, as shown by immunofluorescence staining for gephyrin, neuroligin2 and the $GABA_A$ receptor α2-subunit, as well as VIAAT for GABAergic- and vGluT1 for glutamatergic presynaptic terminals. Therefore, synapse formation is a cell autonomous process independent of the transmitter provided presynaptically. To foster GABAergic innervation, EGFP-expressing GABAergic neurons were seeded onto these cultures at two different developmental stages, at the onset and during ongoing phases of synaptogenesis, respectively. These experiments revealed that GABAergic terminals prevent the formation of mismatched synapses when introduced early, but do not eliminate existing mismatched synapses when introduced after their formation, suggesting a competitive mechanism for interaction with preformed postsynaptic sites. Furthermore, the appearance of GABAergic postsynaptic clusters apposed to both, a GABAergic and glutamatergic terminal suggested an attraction of presynaptic terminals onto such pre-formed postsynaptic clusters.

The question, which proteins are important to induce and maintain postsynaptic clusters of GABAergic proteins was addressed *in vivo* by immunohistochemical analysis in mutant mice. First, we analyzed mice deficient for collybistin, a protein which has been proposed to be important for gephyrin clustering. In Purkinje cells of collybistin-deficient animals, gephyrin clustering is impaired during development until adulthood. Nevertheless, $GABA_A$ receptors and neuroligin2 clustered appropriately at dendritic sites apposed to GABAergic presynaptic terminals suggesting that neither

collybistin nor gephyrin are essential for clustering of these postsynaptic proteins and the formation of GABAergic synapses.

The influence of the $GABA_A$ receptor in formation and stability of GABAergic synapses was investigated in the ventrobasal complex of the thalamus in mice carrying a targeted deletion of the $GABA_A$ receptor $\alpha 1$ subunit gene. This project was carried out in collaboration with Prof. Jeremy Lambert, whose group performed electrophysiological recordings in parallel to our morphological analysis. In thalamic relay cells of the ventrobasal complex, during the second postnatal week $GABA_A$ receptors containing the $\alpha 2$-subunit are replaced by $\alpha 1$-subunit containing receptors, which mediate synaptic inhibitory transmission in the ventrobasal complex of adult animals. In newborn $\alpha 1$-deficient mice, GABAergic synapses form properly, as seen by staining for pre- and postsynaptic proteins as well as electrophysiological recordings. After the second postnatal week, expression of the $\alpha 2$-subunit containing receptors is down regulated and mutant mice become devoid of any synaptic $GABA_A$ receptor in VB neurons. The developmental loss of $GABA_A$ receptor expression leads to disruption of gephyrin clustering and the synapses are silenced. In contrast, presynaptic terminals remain in the same density and size as in wildtype animals. Ultrastructural analysis of the remaining presynaptic terminals revealed that they form specialized non-synaptic junctions with dendrites of relay neurons, constituting potential neurotransmitter release sites maintaining tonic inhibition of extrasynaptically located receptors in the VB of these mice. These results indicate that functional transmission is required for maintenance of synaptic sites, but that presynaptic terminals do not degenerate after synapse disruption, but engage in different contacts with target cells

Taken together, these results indicate a preeminent role of postsynaptic elements in formation and maintenance of GABAergic synapses. None of the proteins investigated here is essential for clustering of $GABA_A$ receptors at the postsynaptic density, but there is a hierarchy in their interaction, with neuroligin2 appearing to play a key role in determining the location of GABAergic synapses, and gephyrin depending on $GABA_A$ receptors for remaining clustered postsynaptically.

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I. General Introduction

1. Synaptic transmission in the brain

In the central nervous system several billions of neurons are communicating in a highly organized manner. A neuron carries information along its axon through electrical impulses generated by cell membrane potential changes, called action potentials. Signal transduction from one cell to the other is then conducted at highly specialized connections named synapses (from Greek συναψις = connection). There are two types of synapses; electrical- and chemical synapses. Electrical synapses between two adjacent neurons are comprised of gap-junction channels which serve as direct conduits between their cytoplasm and thus allow bidirectional communication. Electrical synapses show virtually no delay in signal transmission and are thought to play a role in network synchronization or in neural systems that require the fastest possible response, such as defensive reflexes (Sohl et al., 2005). In contrast, the chemical synapse allows a more specialized and complex way of neuron-neuron communication. There, postsynaptic receptors on the receiving cell are activated through presynaptically released neurotransmitters. The arrival of an action potential at a presynaptic terminal causes an opening of voltage-gated calcium channels, calcium influx into the terminal, and a calcium-dependent fusion of synaptic vesicles containing the neurotransmitter, with the membrane. Thus, neurotransmitters are released into the synaptic cleft and can activate metabotropic and ionotropic receptors at the postsynaptic membrane. Ionotropic receptors form an ion channel pore, which is selective for certain types of ions, whereas metabotropic receptors mediate their action indirectly through G-protein-coupled signaling cascades. Depending on the neurotransmitter and the type of receptor, activation results either in a depolarization or a hyperpolarization of the postsynaptic membrane.

This communication typically is unidirectional, albeit released neurotransmitter molecules can also activate presynaptically located receptors and induce feed-back signals to promote fine-tuning changes of transmitter release characteristics. At chemical synapses, the propagation of signals can be amplified or attenuated. This is regulated by transmitter

release properties at the presynaptic side and by ion channel number and opening kinetics at the postsynaptic membrane (Shepherd, 2004). Thus it is not surprising that chemical synapses act as the major sites of action for psychoactive drugs such as antidepressants, antipsychotics, narcotics, anxiolytics, and antiepileptics (Cooper et al., 2003).

Ion channels conduct ions across the cell membrane at extremely fast rates, which in turn causes rapid changes in the membrane potential. The flow of charge across the membrane depends on the net charge of the ions, the ion-selectivity of the activated channels, and the electrochemical gradient of the ions passing through. The driving force for an ion to cross the membrane is defined by the difference of the actual membrane potential from the reversal potential of the ion, i.e. the potential, when the electrochemical gradient is at equilibrium (McCormick, 2004).

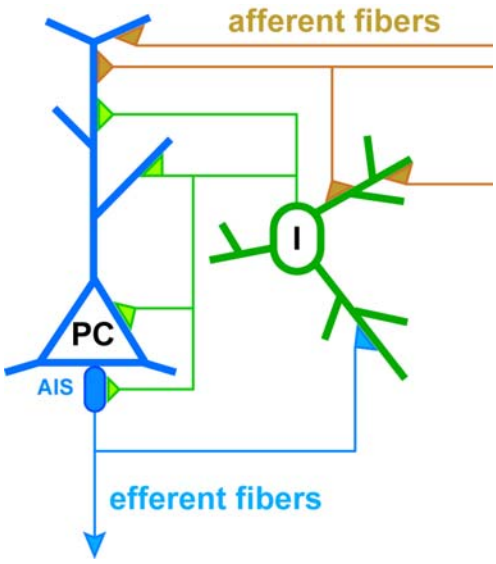

Figure 1

Schematic representation of a neuronal circuit: principal cells (**PC**, most commonly glutamatergic cells, blue) receive synaptic input from afferent fibers (brown, mostly glutamatergic axons), generate action potentials at the axon initial segment (**AIS**) and project to other brain regions. Interneurons (**I**) represent inhibitory neurons projecting within the brain region and are normally GABAergic. Feed-forward inhibition onto a principal cell is mediated by interneurons, which receive input of collaterals of the afferent fibers. In contrast, feed-back inhibition results from synaptic input of an interneuron, which in turn is innervated by a back-projecting axon of the same principal cell.

At rest, the membrane potential of neurons is negative, and the driving force of sodium and calcium ions is directed inwards. Opening of sodium- and calcium-selective ligand-gated ion channels leads to influx of positive charge and a depolarization of the surrounding membrane, driving the membrane potential closer to the threshold necessary for the generation of an action potential. Thus, depolarizing synapses are considered to be excitatory and transmitter release (in the majority of synapses, glutamate) results in the generation of an excitatory postsynaptic potential (EPSP). Apart from depolarizing the

membrane potential, a more important role of postsynaptic calcium influx is attributed to signaling cascades, activated in a calcium dependent manner. Calcium is thought to be the most important second messenger regulating plasticity at pre- and postsynaptic sites.

On the other hand, release of neurotransmitters in inhibitory synapses (most commonly γ-aminobutyric acid (GABA) or glycine) leads to activation of channels selective for chloride ions. Inward directed chloride currents result in a hyperpolarization of the membrane and generation of an inhibitory postsynaptic potential (IPSP). A hyperpolarized membrane remains less excitable and thus the formation of an action potential is inhibited (McCormick, 2004).

In adult neurons at rest, the membrane potential is close to the reversal potential of the inhibitory conductance. Therefore, upon channel opening, the ion flux is rather minimal, but the increased conductance of the membrane leads to a so called "shunting" effect, which reduces the membrane depolarizing effect of concurrent excitatory events of adjacent synapses by virtually clamping the potential close to the reversal potential (Mann and Paulsen, 2007).

In a neuron, the summation of excitatory and inhibitory input at the axon initial segment (Figure 1) determines whether the membrane potential passes the threshold for firing of an action potential. The spatial and temporal integration of excitatory and inhibitory input along the dendrites and soma of a neuron is critical to control its output. Whereas excitatory synapses are thought to mainly mediate network activity, the major role of inhibitory neurons (mostly interneurons, see Figure 1) is assigned to synchronize neuronal network oscillations (Somogyi and Klausberger, 2005). Network oscillations in the cortex have been shown to vary with the behavioral state in animals, suggesting an implication in cognitive processes. Nevertheless, their exact role still remains elusive (Sejnowski and Paulsen, 2006). In a neuronal network, highly organized interconnections between excitatory and inhibitory neurons and exact positioning of each type of synapses are prerequisites for precise rhythmic activity and spike timing. These properties require a precise signaling for cell migration, neurite outgrowth and guidance, and localized induction of synapse differentiation during development.

2. Development of GABAergic transmission

GABA-mediated signaling has been identified as an important factor during key steps of brain development. During early cortical development, functional $GABA_A$ receptors are expressed in neuronal precursor cells in the neocortical proliferative zone (LoTurco et al., 1995; Owens et al., 1996). During migration, synaptic integration and maturation of newly born neurons, the subunit composition of $GABA_A$ receptors as well as its channel kinetics undergo drastic changes, reflecting the involvement of $GABA_A$ receptor signaling in these different developmental steps (Laurie et al., 1992b; Owens et al., 1999). In migrating newborn neurons, $GABA_A$ receptors are not clustered and GABA is considered to have rather trophic functions. When the cells reach their target location, further differentiation of outgrowing neurites and formation of GABAergic and glutamatergic synapses is initiated and GABA action is thought to mediate synaptic transmission.

In developing neurons, GABA first exerts excitatory actions which then turn to inhibitory actions during synapse maturation. The action of GABA depends on the chloride electrochemical gradient across the membrane, which in turn is determined by the developmentally regulated expression of chloride transporters. The K^+-Cl^- cotransporter 2 (KCC2) extrudes chloride ions whereas the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) transports chloride ions intracellularly. In the hippocampus, expression of NKCC1 in immature neurons undergoes a developmental shift in favor to expression of KCC2 in adult neurons. Thus, at resting membrane potential, the electrochemical gradient of chloride changes the ion driving force from an outward to an inward direction, resulting in a switch of excitatory to inhibitory action of GABA. GABA-induced depolarization causes an opening of postsynaptic voltage-gated calcium channels and subsequent calcium influx. Downstream signaling of calcium then activates KCC2 expression and cell maturation (Owens et al., 1996; Ben-Ari, 2002). Even in adult neurogenesis, there is a shift in NKCC1 to KCC2 expression during development of newborn neurons, and GABA-induced depolarization has been shown to regulate their dendritic development and synaptic integration (Ge et al., 2006).

In mammals, synaptogenesis of glutamatergic and GABAergic synapses occurs mostly postnatally (Blue and Parnavelas, 1983; Miller, 1986). It has been shown in the developing

hippocampus that the first synapses established are GABAergic (Tyzio et al., 1999) and that the network of interneurons forms before that of the principal cells, following the same sequence of first GABAergic and later glutamatergic synapse formation (Ben-Ari, 2002; Hennou et al., 2002). This suggests that GABAergic synapses play an important role during early phases of synapse formation. It is even proposed that GABAergic interneurons could control a network-driven pattern at an early developmental stage to initiate more complex neuronal circuits (Ben-Ari et al., 2004).

During synaptogenesis, the $GABA_A$ receptor composition undergoes maturational changes. Thus, in several brain regions a swap in $GABA_A$ receptor subunit composition occurs in both, extrasynaptic as well as synaptic receptors (Laurie et al., 1992b; Paysan et al., 1994; Dunning et al., 1999). In particular, in specific layers of the primary somatosensory and visual cortex as well in the ventral basal complex of the thalamus a switch from the α2-subunit to α1-subunit expression occurs during the period of synaptogenesis, indicating a potential role of $GABA_A$ receptor composition in synapse maturation processes (Fritschy et al., 1994; Paysan et al., 1994).

All together, GABAergic signaling might be crucial for proper development of synaptic circuits, and distinct $GABA_A$ receptor subunits govern different steps during development. Accordingly, targeted gene deletion of the synaptic γ2-$GABA_A$ receptor subunit in mice resulted in an rather normal prenatal development but rapid death after birth. The striking postnatal phenotype may be linked to the onset of synaptogenesis (Gunther et al., 1995). Thus it is of general interest to study $GABA_A$ receptor function during synaptogenesis.

3. Synaptogenesis

The formation of synapses involves a complex series of processes starting with the differentiation of neurons, morphological outgrowth of axons and dendrites, cell-cell contact and site-specific induction of clustering, and assembly of pre- and postsynaptic molecules. To form a fully mature network of interconnected neurons and interneurons, synapses have to be induced at the appropriate developmental stage and at the correct sites. Up to now, more is known about cues for morphological differentiation of neurons, migration, and axon guidance to the target site than about how the formation of a

synapse is initiated and what proteins are involved in these processes. There are at least three steps in synapse formation: (1) clustering of synaptic proteins at the pre- and postsynaptic sites independent of transmitter release and receptor activity, (2) partner recognition among the target axon and dendrite and, (3) synapse maturation or elimination (a process named transient pruning), which very likely depends on receptor activation (Segal, 2001; Craig et al., 2006).

Formation of glutamatergic and GABAergic synapses is likely to involve distinct mechanisms. Glutamatergic synaptogenesis occurs at axonal and/or dendritic protrusions contacting the target neurite, whereas GABAergic synapses form at pre-existing axon-dendrite crossings (Wierenga et al., 2008). The recruitment of pre- and postsynaptic elements are likely to occur independently of each other, while trans-synaptic communication might provide cues for these elements to cluster at the proper sites. This can be concluded from experiments done *in vitro*, where presentation of synaptogenic transmembrane molecules is sufficient for triggering the formation of so-called "hemi synapses". Thus, postsynaptic cell adhesion molecules like neuroligins (NLs; see next section) or synCAM, expressed in non-neuronal cells or coupled to beads, are able to induce clustering of presynaptic proteins and formation of active zones in co-cultured neurons (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004). Likewise, when the presynaptic binding partner of NLs, α- and β-neurexins, are presented to neurons, they induce clustering of proteins in the postsynaptic membrane at the contact sites (Graf et al., 2004; Kang et al., 2008). Furthermore, in immature cultured neurons, several postsynaptic proteins make pre-formed complexes at non-synaptic sites which then can be recruited to synaptic contact zones (Gerrow et al., 2006). To ensure specificity, synaptic cell adhesion molecules may play an important role in acting as signal transducers (Scheiffele et al., 2000; Biederer et al., 2002; Fu et al., 2003; Graf et al., 2004). It has been shown that the orchestration of GABAergic and glutamatergic synapses depends on the expression of NLs, as overexpression of NLs influence the number of GABAergic and glutamatergic synapses (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). Down-regulation of NLs in cultured neurons as well as analysis of NL1-3 knockout mice reveal an involvement of NLs in the balance of excitatory and inhibitory synapses as well as in synapse maturation (Chih et al., 2005; Varoqueaux et al., 2006; Sudhof, 2008).

Very little is understood about how GABAergic postsynaptic proteins are targeted to synaptic sites. It has been shown that for formation of the postsynaptic specialization, clustering of the GABAergic and glycinergic scaffolding protein gephyrin depends on the presence of the $GABA_A$ receptor, but not on its activity (Studler et al., 2002; Schweizer et al., 2003; Kralic et al., 2006). In spite of the lacking $GABA_A$ receptor and gephyrin, GABAergic presynaptic specializations are still present, as seen in cerebellar Purkinje cells of α1-knockout animals, and sometimes form mismatched synapses at dendritic spines (Fritschy et al., 2006). To gain better understanding of the mechanisms and proteins involved in GABAergic synaptogenesis, extensive analysis of protein interaction and signaling at the GABAergic postsynapse is required.

4. Molecular composition of synapses

A chemical synapse is composed of a pre- and a postsynaptic specialization. Ultrastructurally, as seen in electron micrographs, a synapse is recognized by a presynaptic terminal filled with synaptic vesicles apposed to a dendritic structure. It is identified by two electron dense bands at the pre- and postsynaptic membrane (Figure 2 A-B). The active zone of a synapse is defined as the site of the presynaptic transmitter release machinery and corresponds to the thickening of the membrane seen ultrastructurally. It faces exactly the PSD, where postsynaptic receptors are aggregated. Morphologically, GABAergic and glutamatergic synapses are distinguished by their differential electron density of the postsynaptic specialization. At glutamatergic synapses, the PSD appears darker, i.e. electron denser than the presynaptic specialization, resulting in an asymmetric appearance of the synapse. Gray, who first described this characteristic, named it Type I or asymmetric synapse (Figure 2A). Asymmetric synapses are generally found on dendritic spines. GABAergic synapses, in turn, are named Gray Type II or symmetric synapses because of an similar electron density on both, the pre- and postsynaptic specialization (Figure 2B). They are mainly found at dendritic shafts or cell somata (Gray, 1959).

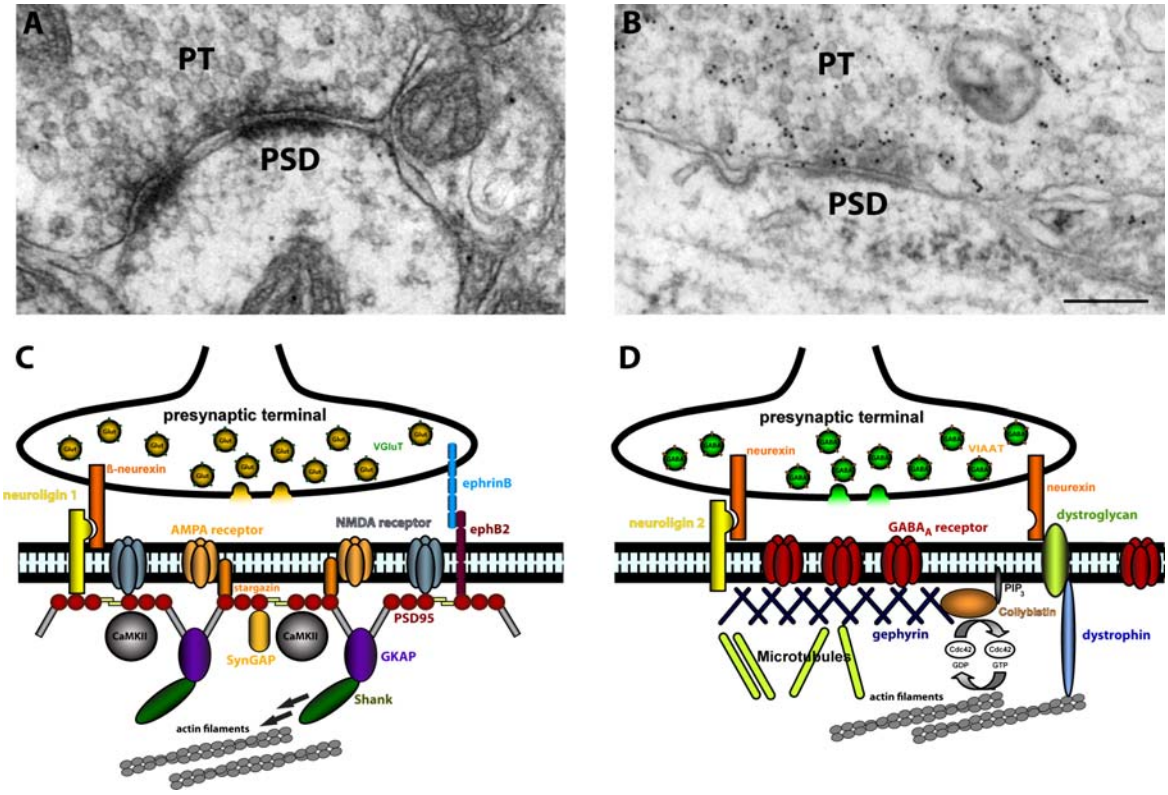

Figure 2

A-B: Transmission electron micrograph of an asymmetric (glutamatergic) (**A**) and a symmetric (GABAergic) (**B**) synapse in the thalamus. The postembedding immunogold labeling of GABA demonstrates the GABAergic presynaptic terminal in **B**, whereas only background levels of gold particles can be found in the presumably glutamatergic terminal in **A**. (PT) presynaptic terminal; (PSD) postsynaptic density; scalebar in B applies for A-B = 200 nm. **C-D**: Schematic representation of a glutamatergic (**C**) and a GABAergic (**D**) synapse. (CaMKII) Ca^{2+}/calmodulin dependent kinase II; (Cdc42) cell division cycle 42; (GKAP) guanylate kinase associated protein; (Glut) glutamate; (PIP_3) phosphatidyl inositol 3-phosphate; (PSD95) postsynaptic density protein 95; (SynGAP) synaptic GTPase activating protein; (VGluT) vesicular glutamate transporter; (VIAAT) vesicular inhibitory amino acid transporter.

As reflected by the similar density in electron microscopy images, glutamatergic and GABAergic presynaptic terminals share a lot of common features. The machinery responsible for calcium-dependent transmitter release including the vesicle associated vSNARE complex proteins (synaptobrevin and the calcium sensor synaptotagmin) and the target membrane proteins of the tSNARE complex (syntaxin1, SNAP25) evolved equally in both types of synapses (Rizo et al., 2006; Tafoya et al., 2006). Specificity is most likely achieved by the many isoforms existing for these proteins (Linial, 1997). Common presynaptic cytoskeletal matrix proteins such as Piccolo and Bassoon as well as cell

adhesion molecules from the cadherin family, which are important for cell-cell interaction and communication are present in both types of synapses (Fenster et al., 2000). Nevertheless, proteins present at the glutamatergic presynaptic terminals but absent in GABAergic synapses like EphrinB or SynCAM are common and most likely contribute to the specificity of the synapse type.

In contrast, enzymes for neurotransmitter synthesis and vesicular transport are unique to the type of terminal. The amino acid glutamate is a metabolite derived from the citrate cycle or taken up into presynaptic terminals as glutamine through glutamine transporters and catalyzed into glutamate by the glutaminase. Glutamate is then transported into the synaptic vesicles via the vesicular glutamate transporters (vGluT1 and vGluT2). In GABAergic terminals, GABA is synthesized from glutamate by the cytosolic enzyme glutamic acid decarboxylase (GAD) and transported into synaptic vesicles by the vesicular inhibitory amino acid transporter (VIAAT). The particular vesicular transporters as well as GAD are specific for the type of neuron and can therefore be used as markers for glutamatergic or GABAergic presynaptic terminals (Kandel et al., 2000).

Postsynaptically, the protein composition of glutamatergic and GABAergic synapses is very distinct. In glutamatergic PSDs, studies based on proteomics revealed a wide range of proteins, including AMPA and NMDA receptors, cell adhesion molecules as neuroligins, ephrinB2, synCAM, the Ca^{2+}/calmodulin dependent kinase II (CaMKII), and scaffolding molecules (postsynaptic density protein 95 (PSD95), GKAP, Figure 2C; (Cheng et al., 2006). In a recent study using high-resolution electron microscopy tomography coupled with antibody labeling, the anatomical structure of glutamatergic PSDs have been visualized. The membrane of the PSD contains NMDA receptors at the centre and AMPA receptors at the periphery; the first submembrane layer is enriched with scaffolding proteins such as PSD95 and a second layer is comprised of Shank and GKAP-family proteins which are further connected to the actin cytoskeleton (Chen et al., 2008).

Less is know about the GABAergic postsynaptic organization. In contrast to glutamatergic postsynapses, which are compartmentalized in dendritic spines, GABAergic PSDs along dendritic shafts are more difficult to access and therefore not readily purified. Apart from GABA receptors, GABAergic synapses contain the NL isoform NL2, the cytoskeleton linker protein dystroglycan, the small rho-like GTPase collybistin, the scaffolding protein gephyrin and many more proteins not identified yet.

At the glutamatergic postsynapse, protein-protein interaction is mostly assured via PDZ-binding domains on scaffolding proteins. PDZ domains are common and well-described modular protein-interaction domains that are specialized for binding to short peptide motifs at the C-terminus of other proteins (Hung and Sheng, 2002). Most proteins of the glutamatergic PSD possess PDZ binding motifs and bind to scaffolding molecules like PSD95, GRIP, and others. Being the archetype of a scaffolding protein, PSD95 includes three PDZ domains and forms a meshwork by self-association. Together, scaffolding proteins and membrane receptors form a web-like protein network to which other cytoplasmic PSD proteins and enzymes can associate (Feng and Zhang, 2009). These interactions also allow to bring interaction partners in close proximity to organize signaling complexes at the postsynaptic membrane. Hence, PSD95 also interacts with a wide variety of cytoplasmic signaling molecules such as CaMKII, thereby contributing to regulatory mechanisms for ion-channel function, synaptic activity, and intracellular signaling. The localization and interaction properties of PSD95 is highly regulated by phosphorylation, palmitoylation for the association with the plasma membrane, and degradation by the ubiquitin-proteasome pathway (Kim and Sheng, 2004).

Major components of GABAergic PSDs do not possess PDZ-binding domains, suggesting a different postsynaptic organization and clustering mechanism there. A likely candidate to govern postsynaptic protein organization and thus act as a scaffolding protein is the multidomain protein gephyrin. Gephyrin is a major component of inhibitory postsynapses and is able to auto-oligomerize, potentially providing a scaffold for postsynaptic proteins and an anchor to the cytoskeleton. Several binding-partners of gephyrin, linking it to the actin and microtubule-cytoskeleton have already been identified (Fritschy et al., 2008). Unpublished observations from our lab suggest that gephyrin undergoes several posttranslational modifications, implicating that gephyrin is highly regulated and therefore might mediate signaling effects for GABAergic synapse formation, maintenance and plasticity. Nevertheless, the precise role of gephyrin and many other GABAergic proteins remains elusive and requires deeper investigation. Therefore, this thesis is devoted to gain further insight into the interaction of different GABAergic postsynaptic proteins and their involvement in GABAergic synapse formation and maintenance.

5. Proteins at the GABAergic postsynapse

In order to give a better understanding of the proteins which will be considered in this thesis, this chapter is dedicated to a selection of proteins which are considered to be important in GABAergic synapse formation and maintenance.

5.1. $GABA_A$ receptors

GABA is the main inhibitory neurotransmitter in the brain and acts on ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. The $GABA_A$ receptor is activated by binding of two GABA molecules and forms a channel selective for chloride ions. In adult neurons, $GABA_A$ receptor activation results in a influx of chloride ions and a hyperpolarization of the postsynaptic membrane.

In mammals, 19 $GABA_A$ receptor subunits have been identified: α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3 which are combined together to form a great variety of heteropentameric channels (Barnard et al., 1998). Most $GABA_A$ receptors are assembled in a composition of two α, two β and a γ-subunit; the γ-subunit can be replaced by a δ-, ε-, θ-, π- or ρ-subunit. The vast majority of $GABA_A$ receptors exist in the composition α1-3β2-3γ2 or δ (Chen and Olsen, 2007).

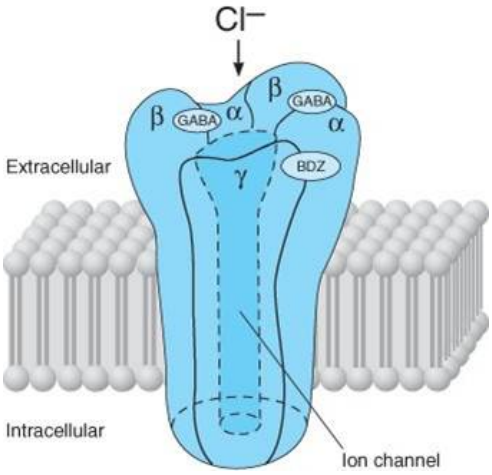

Figure 3:

Schematic representation of the most common $GABA_A$ receptor subunit composition 2α/2β/γ. The two GABA binding sites (**GABA**) between the α and β-subunit as well as the benzodiazepine binding site (**BDZ**) are indicated. Activation of the $GABA_A$ receptor by binding of two GABA molecules results in channel opening and selective permeability for chloride ions.

The molecular heterogeneity of $GABA_A$ receptors is much larger than that of other ligand-gated ion channels, and the expression of $GABA_A$ receptor subtypes exhibits a remarkable region- and neuron-specificity, suggesting that individual types of synapses with different

kinetic and pharmacological properties are present in distinct neuronal circuits (Fritschy and Mohler, 1995; Pirker et al., 2000). Co-expression of α- and β-subunits in non-neuronal cells is sufficient for assembly of functional receptors in the cell membrane, but for full electrophysiological and pharmacological properties, co-expression of either a γ-, δ-, ε-, θ- or π-subunit is essential (Whiting, 1999). The γ2-subunit targets and keeps $GABA_A$ receptors at postsynaptic sites. At synaptic sites, $GABA_A$ receptors mediate fast (phasic) inhibitory neurotransmission. Consequently, a targeted deletion of the γ2-subunit in mice results in a dramatic loss of postsynaptic $GABA_A$ receptors (Essrich et al., 1998) and conditional knockout of the γ2-subunit in three week old mice leads to a sudden loss of postsynaptic $GABA_A$ receptors followed by a lethal epilepsy phenotype (Schweizer et al., 2003). The γ2-subunit is most commonly associated with the α1-, α2-, α3- and α5-subunits (Brunig et al., 2002a).

In contrast, δ-subunit containing receptors are preferentially located at extrasynaptic sites. Receptors located at extrasynaptic sites are activated through GABA spilled over from synaptic release sites and mediate "slow" tonic inhibition. δ-subunits are mostly assembled with α4- and α6-subunits (Nusser et al., 1998).

The different isoforms of the $GABA_A$ receptors are major targets of clinically relevant drugs such as benzodiazepines, barbiturates, and some general anesthetics as well as drugs of abuse like ethanol (Sieghart, 1995; Grobin et al., 1998; Mohler et al., 2002). Benzodiazepines such as diazepam are allosteric agonists of $GABA_A$ receptors and evoke a range of pharmacological actions such as anxiolysis, hypnosis, and sedation. The benzodiazepine binding site is formed by the γ- and α-subunit (Figure 3), and only α1-, α2-, α3-, and α5-subunit containing $GABA_A$ receptors are sensitive to diazepam (Benson et al., 1998; Wingrove et al., 2002). The α-subunit variant determines benzodiazepines-affinity as well as kinetic properties of $GABA_A$Rs (Pritchett et al., 1989; Bosman et al., 2002). By creation of several knock-in mice which carry a point mutation at specific α-subunits disrupting diazepam binding, selective actions of diazepam mediated by distinct $GABA_A$ receptor subtypes could be determined. Thus, sedative effects of diazepam are attributed to $GABA_A$ receptor incorporating the α1-subunit, whereas anxiolytic effects are governed by α2-containing receptors (Rudolph et al., 2001).

Specific $GABA_A$ receptor isoforms show a regional and cell-type specific expression pattern. Purkinje cells in the cerebellum, for example, express $GABA_A$ receptors containing the α1-subunit whereas other α-subunits are absent. In contrast, hippocampal pyramidal cells express high levels of α1-, α2- and α5-subunit along with β1-3 and γ2 (Fritschy and Mohler, 1995; Pirker et al., 2000). α1- and α2-subunit containing receptors are segregated to distinct synapses formed by separate populations of interneurons (Nyiri et al., 2001; Klausberger et al., 2002).

Very little is understood about the mechanisms responsible for the complex pattern of localization and composition of specific types of GABAergic receptors. Further studies on $GABA_A$ receptor interacting proteins during synaptogenesis will yield a better insight into formation and maintenance of GABAergic synapses.

5.2. Gephyrin

Gephyrin is a major component of inhibitory synapses. Originally, gephyrin was co-affinity purified with the glycine receptor and found to directly bind to and anchor glycine receptors to the sub-synaptic cytoskeleton (Prior et al., 1992; Kirsch and Betz, 1995; Meyer et al., 1995). Later, gephyrin was also identified as a major component of GABAergic synapses important for $GABA_A$ receptor clustering (Sassoe-Pognetto et al., 1995; Craig et al., 1996; Essrich et al., 1998). In contrast to the strong binding of gephyrin to the glycine receptor, the demonstration of similar binding properties to $GABA_A$ receptor subtypes has failed so far. A weak interaction of gephyrin with only the α2-subunit of the $GABA_A$ receptor has been reported and their association could be detected only under detergent-free conditions, suggesting a hydrophobic interaction (Tretter et al., 2008). No additional evidence for binding of gephyrin to other $GABA_A$ receptor subtypes has been found yet. This may be due to weak hydrophobic binding properties or due to an unidentified interaction partner promoting indirect binding.

Gephyrin consists of three protein domains: an N-terminal G-domain and an C-terminal E-domain, which embrace a central linker domain. The G- and E-domain share high sequence homology with the bacterial Moco-synthesizing enzymes MogA and MoeA, respectively. In all species, gephyrin is expressed ubiquitously and exerts at least two functions: in most tissues, it acts as an enzyme in the Moco biosynthesis (Feng et al.,

1998) whereas in the brain it appears in addition as a scaffold protein in GABAergic and glycinergic synapses (Prior et al., 1992; Paarmann et al., 2006). Alternative splicing of the 30 exons in the mouse gephyrin gene (Gphn) give rise to a great variety of isoforms. Both, the G- and the E-domain of the full-length P1 isoform (Prior et al., 1992) have been crystallized, and a model of auto-aggregation of these domains has been proposed. In this model, G-domain trimerization and E-domain dimerization would yield a hexagonal mesh-like structure, supporting the concept of gephyrin providing a scaffold for clustering of postsynaptic proteins and anchoring them to the cytoskeleton (Schwarz et al., 2001; Sola et al., 2001; Sola et al., 2004). The role of gephyrin as a scaffolding protein is further supported interaction partners linking it to the cytoskeleton. In particular, gephyrin is known to interact with Mena/VASP and profilin, proteins which are involved in actin filament regulation (Giesemann et al., 2003).

Even though the presence of gephyrin is not essential for clustering of GABAergic postsynaptic proteins, removal of gephyrin strongly affects GABAergic synapse stability and $GABA_A$ receptor function (Kneussel et al., 1999). In hippocampal neuronal cultures, gephyrin down regulation leads to a decrease in $GABA_A$ receptor clusters (Levi et al., 2004; Yu et al., 2007), whereas overexpression of mutant forms of gephyrin with increased tendency to form clusters concomitantly leads to an increase in GABAergic postsynaptic clusters (Lardi-Studler et al., 2007). Nevertheless, the precise role of gephyrin in processes for GABAergic synapse formation and maintenance remains elusive and requires further studies of gephyrin and interacting proteins.

5.3. Collybistin

Collybistin is a brain specific member of the Dbl-family of guanine nucleotide exchange factors (GEF) and specifically acting on the small Rho-like GTPase Cdc42, which in turn is known to regulate the organization of actin filaments (Hall, 1998; Reid et al., 1999). Upon co-expression in non-neuronal cells, collybistin has been shown to directly interact with gephyrin and to induce its submembrane clustering (Kins et al., 2000; Grosskreutz et al., 2001). The protein is expressed in different splice variants, the majority of them harboring an N-terminal *src* homology 3 (SH3)-, a Dbl-homology (DH) domain which also incorporates the catalytic RhoGEF domain, and a C-terminal pleckstrin homology (PH)

domain. The SH3 domain influences collybistin activity and gephyrin cluster size by interaction with unknown binding partners, whereas the PH domain was found to be required for submembrane recruitment of collybistin and gephyrin via phosphatidyl inositol (Kins et al., 2000; Harvey et al., 2004). Interestingly, Cdc42 binding to collybistin is negatively regulated by gephyrin, suggesting competitive binding of gephyrin and Cdc42 to collybistin (Xiang et al., 2006).

Taken together, collybistin seems to have an important role in clustering of gephyrin and other GABAergic postsynaptic proteins *in vitro*. *In vivo*, collybistin-deficient mice show only a mild phenotype and gephyrin and $GABA_A$ receptor clustering is only affected in a region- and cell-specific manner, leaving the exact role of the protein in GABAergic synapse formation still elusive (Papadopoulos et al., 2007).

5.4. Neuroligin 2

NL2 is a member of a family of brain-specific cell adhesion molecules and selectively enriched at GABAergic postsynaptic sites (Varoqueaux et al., 2004). It consists of a large extracellular N-terminal domain which is homologous to serine esterase but lacks an active site, followed by a membrane spanning domain and a C-terminal intracellular domain incorporating a PDZ binding motif. The serine esterase domain binds to presynaptically located α- and β-neurexins (Ichtchenko et al., 1996), linking pre- and postsynaptic specializations together.

All NL isoforms have been implicated in synaptogenesis, as expression of each NL isoform in non-neuronal cells is sufficient for triggering the formation of active zones in axon terminals of co-cultured neurons contacting them (reviewed in Biederer and Stagi, 2008). In addition, its relative expression level determines the number of synapses formed (Chubykin et al., 2007). *In vivo*, in NL deficient mice, it has been shown that NLs are required for proper synapse maturation and brain function, rather than for the initial formation of synaptic contacts (Varoqueaux et al., 2006). Nevertheless, the specificity of synaptic contacts is thought to depend on adhesion molecules acting as signal transducers (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004) and to provide cues to pre-formed pre- and postsynaptic molecular constituents for clustering at appropriate sites

(Gerrow et al., 2006). Therefore, NL2 is an interesting candidate to investigate mechanisms for synapse formation at specific sites.

All together, different proteins which are likely to be involved in processes of GABAergic synapse formation, protein clustering and synapse maturation have been identified. Nevertheless, their exact roles and interaction properties are not well understood and require further investigation.

6. Aim of the thesis

The general aim of this thesis was to gain further insight into mechanisms of GABAergic synapse formation and maintenance, and to determine which proteins are important to ensure clustering of postsynaptic elements. We wanted to identify key players and deduce a hierarchy of protein clustering and subsequent GABAergic synaptogenesis. In particular, we focused on collybistin-dependent clustering of gephyrin and its influence on $GABA_A$ receptors and NL during synaptogenesis, on dynamic effects of changes in $GABA_A$ receptor expression during synapse maturation, as well as the role of presynaptic input onto these processes.

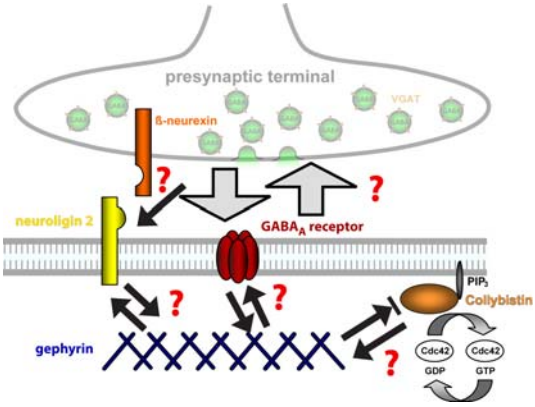

Figure 4:

Model of potential correlations for clustering and maintenance of postsynaptic elements at the GABAergic synapse. The question marks indicate the interactions we want to investigate: (1) how do pre- and postsynaptic elements influence each other? (2) is collybistin-dependent clustering of gephyrin important for $GABA_A$ receptor and NL clustering and (3), how does the absence of the $GABA_A$ receptor influence other synaptic elements?

To address these issues, we chose an *in vitro* approach, allowing us to control the presynaptic input onto hippocampal cultured neurons and to study the synaptic cell-cell communication during early phases of GABAergic synapse development, as well as *in vivo* approaches analyzing mice deficient for one of the proteins of interest. Immunohistochemical methods were used to investigate the following three questions:

(1) What is the influence of GABAergic presynaptic input on the assembly and formation of the postsynaptic density during GABAergic synaptogenesis?

To study the influence of presynaptic GABAergic input during early stages of synaptogenesis, we used an *in vitro* approach. In embryonic day 18 (E18) primary cultured hippocampal neurons, most GABAergic interneurons do not survive the preparation procedure, and thus the vast majority of cells are glutamatergic pyramidal

cells. Therefore, early phases of synaptogenesis happen in the absence of GABAergic input, and GABAergic innervation occurs only at later stages, when the axons of the few interneurons have propagated within the culture (Brunig et al., 2002b). Despite the lack of presynaptic GABAergic input, postsynaptic GABAergic proteins cluster properly and localize apposed to glutamatergic presynaptic terminals where they form "mismatched synapses" (Rao et al., 2000; Brunig et al., 2002b). Exploiting this neuronal culture system lacking early GABAergic input, we established a co-culture system where we control GABAergic innervation by seeding ectopic GABAergic neurons onto these hippocampal cultures at different developmental stages. When GABAergic cells were seeded early, at the onset of synaptogenesis, such co-cultures allowed us to study the influence of GABAergic input before mismatched synapses have formed. In contrast, when the cells were seeded later, after onset of synapse formation, we analyzed the effect of GABAergic innervation onto clustered postsynaptic proteins in already formed mismatched synapses. To understand, whether mismatched synapses are stable entities or only transient contacts, we studied the localization of major components of the GABAergic postsynaptic density at properly matched and mismatched synapses. Using immunocytochemistry, we stained for the $GABA_A$ receptor, gephyrin, and NL2 based at terminals positive for GABAergic (VIAAT) and glutamatergic (vGluT) markers.

(2) Does the absence of collybistin-dependent clustering of gephyrin affect GABAergic synapse formation?

This question was addressed in cerebellar tissue sections of mice deficient for collybistin. It has been shown previously that in Purkinje cells of adult collybistin-knockout mice, clustering of gephyrin is impaired (Papadopoulos et al., 2007). Purkinje cells display features which makes their analysis very suitable to study our question: (1), nearly all $GABA_A$ receptors are assembled in the composition $\alpha 1\beta 2/3\gamma 2$. We therefore can exclude alternative receptors involved in synapse establishment. (2), synapse formation happens exclusively postnatally, allowing easy collection of the tissue at the onset of synaptogenesis, and (3), the cerebellar cortex, which incorporates the cell bodies and dendritic trees of the Purkinje cells, is a highly organized and layered structure, and the outgrowth of Purkinje cell neurites and development of GABAergic innervation by

interneurons is well defined. Therefore, alterations in protein expression are easy to characterize. We compared the distribution of gephyrin, $GABA_A$ receptors and NL as well as presynaptic markers in cerebellar sections of WT and collybistin-knockout mice during developmental stages of onset (postnatal day 7 (P7)), peak (P10) and termination (P20) of GABAergic synaptogenesis.

(3) Once a GABAergic synapse is established, does removal of the $GABA_A$ receptor impair the maintenance of the synapse?

In the rodent ventrobasal complex of the thalamus (VB), GABAergic synapses undergo drastic changes in subunit expression during synaptogenesis (Fritschy et al., 1994). $GABA_A$ receptors containing the $\alpha 2$ subunit are replaced during the third postnatal week by $\alpha 1$ subunit-containing $GABA_A$ receptors, which mediate synaptic inhibitory transmission in the VB of adult animals. In the VB of adult $\alpha_1^{0/0}$ mice, neurons lack any type of synaptic $GABA_A$ receptors, and the postsynaptic clustering of gephyrin is profoundly altered (Kralic et al., 2006). We therefore analyzed the VB of $\alpha 1$-deficient mice during postnatal development and investigated, if, despite of the lack of $\alpha 1$-subunit expression in adult mice, GABAergic synapses are formed during early development, when $\alpha 2$-containing $GABA_A$ receptor are expressed. If GABAergic synapses were effectively formed, we wanted to address the question whether the lack of the $\alpha 1$ subunit affects the disappearance of $\alpha 2$-$GABA_A$ receptors and the formation of gephyrin clusters during postnatal maturation, and whether GABAergic presynaptic terminals are affected by the loss $\alpha 1$-expression in mutant mice. Using immunohistochemistry, we analyzed the distribution of $\alpha 1$- and $\alpha 2$-subunit expression, their co-localization with gephyrin, and if these clusters appear apposed to terminals marked for GABAergic presynaptic proteins from the stages of the $GABA_A$ receptor subunit expression in the third postnatal week change to adulthood. To observe potential changes in morphology, we analyzed synapses at the ultrastructural level using transmission electron microscopy combined with immunohistochemical staining for gephyrin and GABA. In addition, in a collaboration with the group of Dr. Jeremy Lambert and Dr. Delia Belelli at the University of Dundee, UK, we analyzed the electrophysiological properties of VB relay neurons in the $\alpha_1^{0/0}$ mice.

II. Results

1. Study I: Postsynaptic mechanisms influence the formation of GABAergic synapses in hippocampal cultured neurons

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Submitted

1.1. Abstract

Synapse formation involves bidirectional signaling between pre- and postsynaptic elements for recognition of contact sites and differentiation of the synaptic apparatus. Hippocampal neurons cultured in absence of GABAergic cells form apparent "mismatched" synapses recognized by clusters of $GABA_A$ receptors and gephyrin at sites contacted by glutamatergic terminals. Therefore, initial steps of synapse formation and recruitment of postsynaptic proteins are independent of neurotransmission *in vitro*. Here, we investigated using immunofluorescence analysis the formation of GABAergic synapses in rat low density hippocampal cultures upon introducing ectopic EGFP-positive GABAergic cells at different stages of maturation. We report that neuroligin2 – a postsynaptic protein targeted to GABAergic synapses *in vivo* and interacting with presynaptic neurexin isoforms – is colocalized with gephyrin and $GABA_A$ receptors in both matched and mismatched synapses *in vitro*. GABAergic neurons introduced at the onset of synaptogenesis (3 days-*in-vitro*) largely prevented mismatched synapse formation, suggesting a competitive advantage of GABAergic terminals over glutamatergic terminals for recruiting GABAergic postsynaptic proteins. When introduced after 6 days-*in-vitro,* GABAergic neurons successfully innervated hippocampal cells, albeit without eliminating established mismatched synapses. GABAergic axons did not replace glutamatergic axons in mismatched synapses but formed *de novo* synapses. In both conditions, however, numerous gephyrin clusters were apposed simultaneously to GABAergic and glutamatergic terminals, suggesting that nascent pre-formed postsynaptic sites attract presynaptic axons to initiate synapse formation independently of the neurotransmitter. Altogether, these results point to an inductive role of postsynaptic protein clusters containing neuroligin2 and gephyrin for synaptogenesis in hippocampal neurons. Neuroligin2 might be responsible for trans-synaptic signaling required to trigger synapse differentiation.

1.2. Introduction

The formation of synapses requires bidirectional interactions between pre- and postsynaptic elements (Waites et al., 2005; Craig et al., 2006). *In vitro*, the presentation of synaptogenic transmembrane molecules to neurons is sufficient for triggering the formation of "hemi-synapses". Thus, recombinant expression of postsynaptic cell adhesion molecules, such as neuroligin (NL), synaptic cell-adhesion molecule or fibroblast growth factor 22, in non-neuronal cells co-cultured with neurons induces clustering of presynaptic proteins and formation of active zones in axon terminals contacting them (reviewed in Biederer and Stagi, 2008)). Conversely, presentation of the presynaptic binding partner of NLs, α- and β-neurexins, to neurons induces morphological differentiation of the postsynaptic density (PSD) and clustering of postsynaptic proteins at the contact sites (Graf et al., 2004; Kang et al., 2008). *In vivo*, NLs are not essential for synapse formation, as shown in NL1-3 knockout mice. Rather, they play a role in the balance of excitatory and inhibitory synapses and in synapse maturation (Chih et al., 2005; Varoqueaux et al., 2006; Sudhof, 2008). NL1 and NL2 are segregated between glutamatergic and GABAergic synapses, respectively (Song et al., 1999; Varoqueaux et al., 2004); their relative expression level determines the number of each type of synapses formed (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). The specificity of synaptic contacts is thought to depend on adhesion molecules acting as signal transducers (Scheiffele et al., 2000; Biederer et al., 2002; Fu et al., 2003; Graf et al., 2004) and to provide cues to pre-formed pre- and postsynaptic molecular constituents for clustering at appropriate sites (Gerrow et al., 2006). In turn, the expression levels of functional postsynaptic receptors were shown to determine the rate of synapse formation *in vitro* and *in vivo* (Fang et al., 2006; Deng et al., 2007; Patrizi et al., 2008).

The formation of glutamatergic and GABAergic synapses is likely governed by distinct mechanisms, with glutamatergic synapses developing at contact sites of axonal and/or dendritic protrusions with the target neurite whereas GABAergic synapses form at pre-existing axon-dendrite crossings (Wierenga et al., 2008). While transcription factors regulated by activity-dependent mechanisms, such as Npas4, have been implicated in GABAergic synapse formation (Lin et al., 2008), little is understood about the mechanisms of sorting, targeting, and clustering of GABAergic postsynaptic proteins to synaptic sites.

GABAergic synapse formation and maintenance can occur in the absence of $GABA_A$ receptors, as shown in $\alpha 1$ subunit knockout ($\alpha_1^{0/0}$) mice (Fritschy et al., 2006; Patrizi et al., 2008; Peden et al., 2008). On Purkinje cells, such synaptic contacts are ultrastructurally indistinguishable from functional synapses formed in wildtype mice, although numerous GABAergic terminals make mismatched synapses on dendritic spines with asymmetric postsynaptic densities (PSD). In $\alpha_1^{0/0}$ mice, NL2 clusters form selectively at symmetric (i.e. properly matched) but not asymmetric (i.e. mismatched) synapses, suggesting that NL2 clustering is restricted to specific cell surface domains, such as dendritic shafts (Patrizi et al., 2008). In contrast to NL2, postsynaptic clustering of the scaffolding protein gephyrin depends on presence of $GABA_A$ receptors, probably through an interaction involving the $\gamma 2$ subunit (Studler et al., 2002; Schweizer et al., 2003; Kralic et al., 2006; Peden et al., 2008). Conversely, when neurons are grown *in vitro* in the absence of GABAergic innervation, they readily form "mismatched" synapses recognized by $GABA_A$ receptor and gephyrin clusters being apposed to glutamatergic terminals (Rao et al., 2000; Brunig et al., 2002b; Studler et al., 2002). Taken together, these results suggest that sorting and targeting of postsynaptic proteins to the correct synapse can occur without neurotransmitter-mediated cues.

The aim of this study was to gain further insight into the role of cell-cell communication and neurotransmission for GABAergic synaptogenesis. We investigated a co-culture system comprising hippocampal neurons grown at low density to induce formation of "mismatched synapses" and ectopic GABAergic cells isolated from the striatum of neonatal glutamic acid decarboxylase 67-GFP (GAD67-EGFP) mice (Tamamaki et al., 2003) and introduced at selected stages of maturation *in vitro*. These co-cultures were analyzed to determine whether "mismatched" synapses occur in neurons innervated early on by GABAergic terminals and whether they are stable entities or only transient contacts between glutamatergic terminals and preformed GABAergic PSDs. Using immunofluorescence with markers of glutamatergic and GABAergic pre- and postsynaptic sites (vesicular glutamate transporter 1, vGluT1; vesicular inhibitory amino acid transporter, VIAAT; $GABA_A$ receptor $\alpha 2$ subunit; gephyrin; NL2), we observed that GABAergic innervation at the onset of synaptogenesis largely prevents mismatched synapse formation, whereas delayed GABAergic input does not eliminate already formed mismatched synapses. Unexpectedly, we frequently observed gephyrin puncta apposed to

both, GABAergic and glutamatergic terminals in all co-cultures examined. These "dually innervated" postsynaptic sites suggest an inductive role of preformed postsynaptic clusters for the formation of GABAergic synapses.

1.3. Experimental procedures

Animals

Hippocampal primary cultures were prepared from Wistar rat embryos (RCC, Itingen, Switzerland) as described previously (Brunig et al., 2002b). Striatal ectopic cells were obtained from GAD67-EGFP knock-in mice (for characterization see Tamamaki et al., 2003) bred on a heterozygous background by crossing with C57BL/6J mice. Neonatal heterozygous pups were identified by illumination with a blue fluorescent light source revealing intense EGFP in the brain. All experiments were performed according to the international guidelines on animal care and use and were approved by the cantonal veterinary office of Zurich.

Cell cultures

Pregnant dams were anesthetized with ether and killed by cervical dislocation. Embryos (E18) were rapidly taken out and put on ice. The hippocampus was dissected on ice and incubated in PBS containing 1 mg/mL bovine serum albumin, 10 mM glucose (Sigma, St Louis, MO, USA), 0.5 mg/mL papain (Sigma) and 10 μg/mL DNAse-I (Sigma) for 15 min at 37°C. Cells were then dissociated mechanically with a fire-polished Pasteur pipette and re-suspended in DMEM (Gibco, Invitrogen, San Diego, CA, USA) containing 10% fetal calf serum (FCS, Invitrogen). They were plated on poly-L-lysine (Sigma) coated glass coverslips (18 mm, thickness 0.17 ± 0.02 mm, Assistant, Germany) at a density of 20'000 cells per coverslip and kept for one hour at 37°C in a 5% CO_2 humidified incubator. Astrocyte cultures prepared from P0 rat cortex were plated in advance on 12-well dishes and cultivated in DMEM containing 10% FCS to generate a feeder layer. The glial cell medium was exchanged with neurobasal medium (Gibco) containing 20 μl/mL B27-supplement (Gibco), 2 mM L-glutamine (Gibco) and 50 μg/mL gentamicin and the neuron coverslips were placed facing the feeder layer. Cultures were kept at 37°C in a 10% CO_2 humidified incubator and analyzed after 3 to 18 days-in-vitro (DIV).

For hippocampal-striatal co-cultures, the striatum of P0 or P1 GAD67-EGFP mice was dissected according to (Ventimiglia and Lindsay, 1998) on ice and dissociated as described

above for the hippocampus. Striatal cells were seeded onto coverslips carrying hippocampal neurons after 3 or 6 DIV. On the following day, the coverslips were turned upside-down again onto the glial feeder layer and kept in culture for another 6 days, yielding either 3+6 or 6+6 DIV cultures for analysis.

Immunohistochemistry

The following antibodies were used: affinity-purified guinea pig antiserum against $GABA_A$ receptor α2-subunit (0.5 μg/mL; raised in-house, for characterization see Fritschy and Mohler, 1995), mouse anti-gephyrin mAb7a (1:400; Synaptic Systems, Göttingen, Germany), rabbit anti-NL2 (1:2000; gift from Frédérique Varoqueaux, Göttingen; characterized in Varoqueaux et al., 2004), mouse anti-VIAAT (1:2000; Synaptic Systems), affinity purified rabbit antiserum against VIAAT (1:1000; Synaptic Systems), guinea pig antiserum against vGluT1 (1:10'000; Chemicon, Temecula, CA, USA) and rabbit antiserum against vGluT1 (1:1000; Synaptic Systems). The specificity of the VIAAT and vGluT1 antibodies was verified by double immunofluorescence staining with the two antibodies raised in different species.

For detection of the $GABA_A$ receptor α2 subunit, living cultures were incubated for 90 minutes at room temperature with the antibody diluted in 25 mM HEPES buffer containing 30 mM glucose, 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 μM Glycine and 500 nM tetrodotoxin (Latoxan, Valence, France), as described in Brunig et al., (2002b), and washed in the same solution before fixation. All other primary antibodies were applied after fixation.

Cells were fixed in 150 mM sodium phosphate buffer containing 4% paraformaldehyde (EMS, Hatfield, PA, USA), pH 7.4 for 15 minutes and permeabilized in PBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 for 3 minutes at room temperature. Primary antibody incubation was performed in PBS containing 10% NGS for 90 minutes at room temperature in a moist chamber. The cultures were washed in PBS and incubated with a mixture of secondary antibodies raised in goat and coupled to Cy3 and Cy5, respectively (1:500 and 1:200, Jackson ImmunoResearch, West Grove, PA, USA) or Alexa488 (1:1000, Molecular Probes, Eugene, OR, USA) in PBS with 10% NGS for 30

minutes at room temperature. Coverslips were washed again with PBS and mounted on glass slides with fluorescent mounting medium (DAKO, Carpinteria, CA, USA).

Image analysis

All experiments were analyzed by confocal laser scanning microscopy (Zeiss, LSM 510 Meta, Jena, Germany) using a 100x Plan-Apochromat oil immersion objective (N.A. 1.4; pixel size = 0.03 μm^2). The pinhole was set to 1 Airy unit and different color channels were scanned sequentially. The acquisition settings were adjusted to cover the whole dynamic range of the photomultipliers. For display, images were processed with the imaging software Imaris (Bitplane, Zurich, Switzerland) and ImageJ (NIH, Bethesda, MD, USA). Images from a single confocal section with three channels were overlaid (maximum intensity projection), and the background was subtracted if necessary.

For quantification, raw data images of single hippocampal cells were used. Colocalization of NL2 with gephyrin in relation to vGluT1-positive glutamatergic terminals was assessed in triple immunofluorescence staining of 18 DIV neurons, using a colocalization algorithm in ImageJ. The localization of gephyrin clusters in matched and mismatched synapses was quantified in co-cultures at 3+6 and 6+6 DIV, using immunofluorescence staining for gephyrin, VIAAT and vGluT1. Because VIAAT-positive terminals appeared as brightly stained puncta, they could be readily distinguished in EGFP-positive axons even when using a fluorochrome with an overlapping emission spectrum, allowing the distinction of four markers in triple staining experiments. From all color channels, a threshold cut-off of 40-45% of the maximum intensity was applied to distinguish clusters of synaptic proteins from the diffuse staining of the surrounding neurites. The total number of gephyrin clusters in the cell was counted in the thresholded image by macro-based automated batch processing using ImageJ, using a minimal size of 2 pixels, corresponding to 0.06 μm^2. Apposition to presynaptic terminals was defined as a direct contact or overlay between a gephyrin cluster and a VIAAT- or vGluT1-positive structure. In the co-culture experiments, the number of matched (gephyrin apposed to VIAAT), mismatched (gephyrin apposed to vGluT1), dually innervated (gephyrin apposed to both VIAAT and vGluT1), and non-innervated (extrasynaptic) gephyrin clusters was determined in proportion of the total

number of gephyrin clusters. All results are derived from three independent culture batches and are presented as mean ± SD, derived from 10 neurons per batch.

Statistical analysis was performed to test whether the proportion of matched, mismatched, dually innervated, and extrasynaptic gephyrin clusters differ between 3+6 and 6+6 DIV co-culture experiments. As the total number of gephyrin clusters was significantly different between the two stages, we analyzed arcsin-transformed relative values using ANOVA with repeated measures on the same subjects followed by *t*-test with separate variances for post-hoc mean comparisons. Finally, the size of matched and mismatched gephyrin clusters was quantified separately in 3+6 and 6+6 DIV cultures and tested for significant differences using cumulative distribution analysis (Kolmogorov-Smirnov).

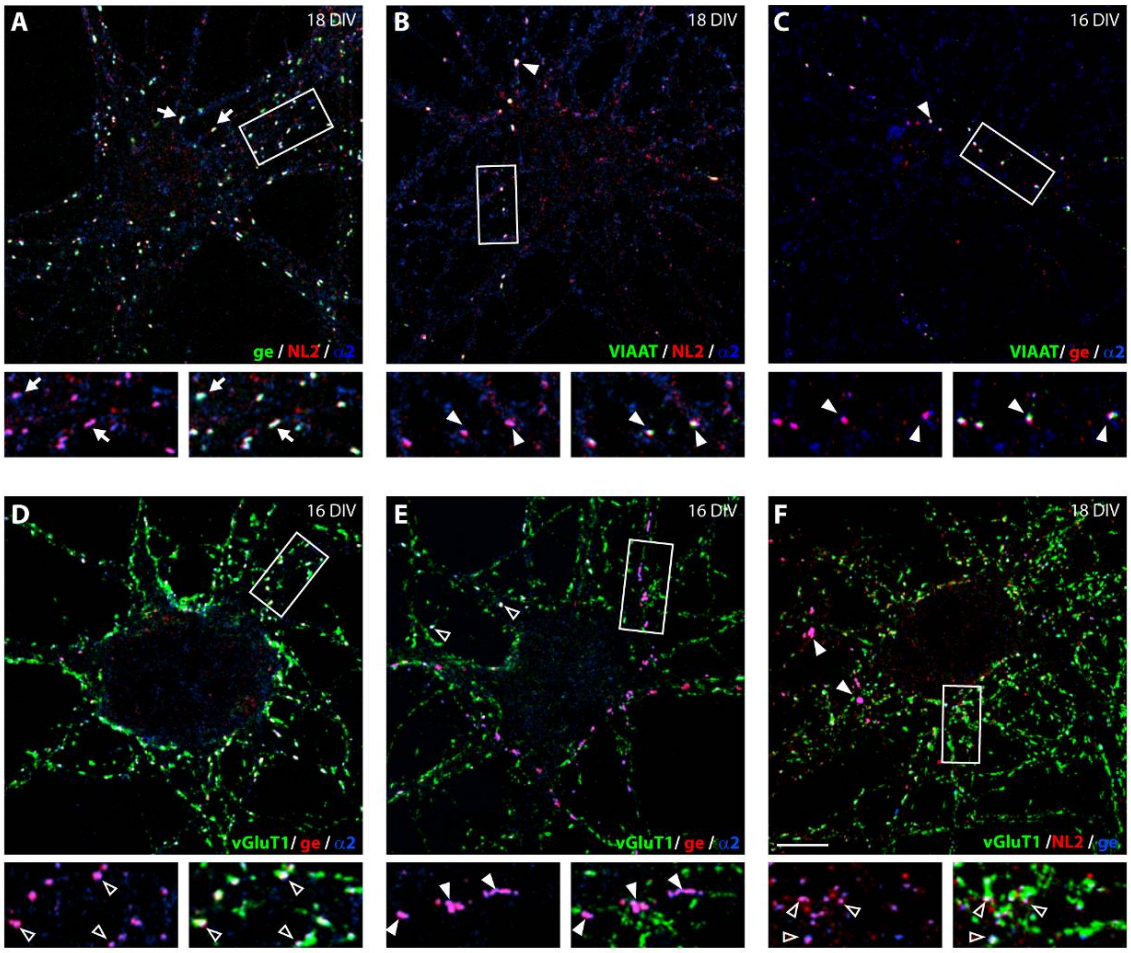
1.4. Results

Lack of GABAergic innervation leads to mismatched apposition of postsynaptic GABAergic proteins and glutamatergic terminals

Gephyrin and NL2 are two markers of GABAergic synapses colocalized with the $GABA_A$ receptor α2 subunit in clusters formed on the soma and dendrites of cultured hippocampal neurons, as seen by immunofluorescence staining after 16-18 days-in-vitro (DIV, Figure 1A). At this stage, the number of gephyrin- and NL2-immunoreactive clusters per neuron was 211 ± 85 and 288 ± 125 (mean ± SD), respectively. Staining for VIAAT revealed that only a subset of postsynaptic GABAergic clusters were apposed to a GABAergic terminal (Figure 1B-C). In these cultures, GABAergic axons typically were long and straight, making isolated contacts "en passant" while crossing over the dendrites and soma of presumptive pyramidal cells. Clusters immunoreactive for NL2, gephyrin and the α2 subunit at these sites appeared large and intensely stained (Figure 1B-C). In contrast to GABAergic axons, staining for vGluT1 revealed an extensive network of glutamatergic terminals outlining the soma and proximal dendrites of most cells (Figure 1D-F). Examination at high magnification confirmed the formation of mismatched synapses (Rao et al., 2000; Brunig et al., 2002b), as seen by the close apposition between vGluT1-positive (glutamatergic) terminals and clusters immunoreactive for GABAergic postsynaptic proteins (α2 subunit, gephyrin, NL2) (Figure 1D, 1F, insets). These postsynaptic clusters were smaller than those apposed to GABAergic terminals. Thus, the dual innervation of cells was evidenced by the presence of small gephyrin/α2 clusters on all dendrites and large clusters aligned along straight virtual lines (Figure 1E); vGluT1-positive terminals typically were not in close contact with these presumptive GABAergic postsynaptic sites. In agreement with our previous results (Studler et al., 2005), gephyrin-immunoreactive clusters were

Figure 1

Co-clustering of major GABAergic proteins at glutamatergic and GABAergic terminals, as seen by immunofluorescence staining and confocal laser scanning microscopy of immature hippocampal neurons in culture. Each panel depicts a single confocal layer. The boxed area is displayed at higher magnification in color-separated images below each panel. **A**:



Immunoreactivity of gephyrin (ge; green), NL2 (red), and the $GABA_A$ receptor α2-subunit (blue) is nearly always found co-clustered (arrows) on the dendrites and soma of cultured hippocampal cells after 18 DIV. **B, C**: In hippocampal cells receiving some GABAergic innervation, evidenced by VIAAT-positive GABAergic terminals (green), clusters double-labeled for NL2 (**B**, red) or gephyrin (**C**, red) and the α2-subunit (blue), are localized apposed to these terminals (pink clusters), forming properly matched synapses, which appear white in the overlay (filled arrowheads). **D**: All neurons receive a dense innervation by vGluT1-positive terminals (green). The majority of gephyrin- (red) and α2 subunit-positive (blue) clusters (pink) is found apposed to vGluT1-positive terminals, where they form mismatched synapses (white; empty arrowheads). **E**: In most cells, large gephyrin (red) and α2-positive (blue) clusters can be found along an imaginary line, which are devoid of vGluT1-staining (green). They most likely represent correctly matched synapses in a cell which is innervated by a GABAergic axon. Note that only few mismatched synapses (white; empty arrowheads) remain on other dendrites. **F**: Similarly to the α2 subunit, NL2 staining (red) is found co-clustered with gephyrin (blue), forming large and probably correctly matched clusters (full arrowheads) as well as small clusters apposed to vGluT1-staining (green) at mismatched synapses (empty arrowheads). Scale bar for A-F = 10 μm.

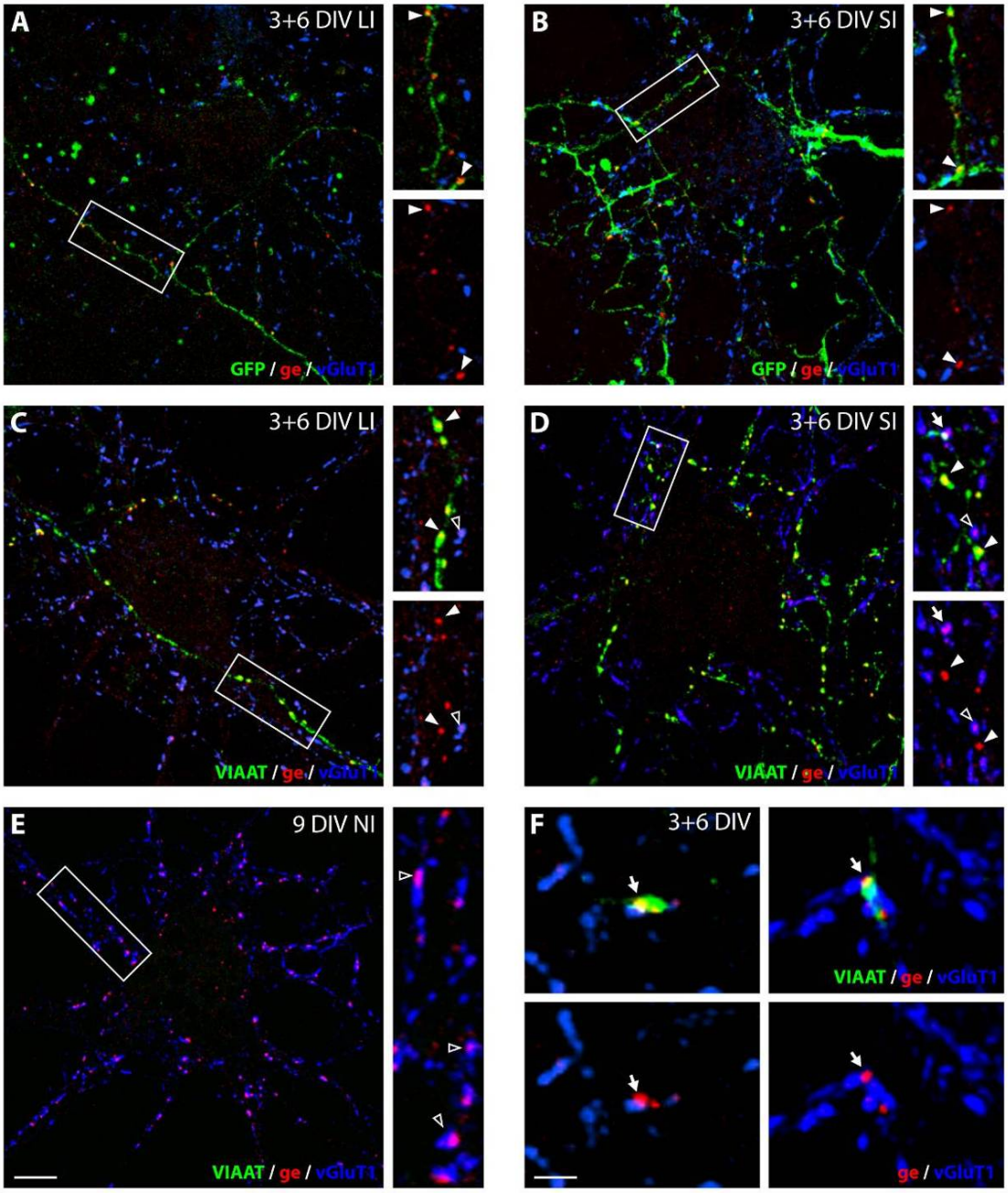
systematically co-localized with the α2 subunit, regardless of whether they formed matched or mismatched synapses (Figure 1A-C). Quantification of gephyrin/NL2 colocalization revealed that 80% of gephyrin clusters and 65% of NL2 clusters were colocalized with each other. Gephyrin clusters devoid of NL2 were located either at extrasynaptic sites (65%) or apposed to vGluT1-positive puncta (35%). The opposite proportion was found for NL2 clusters devoid of gephyrin (33% apposed to vGluT1 terminals and 67% extrasynaptic).

Formation of GABAergic synapses in striato-hippocampal co-cultures

Colocalization of gephyrin, NL2, and α2 subunit in clusters facing vGluT1-positive terminals suggests that GABAergic PSD can form without instructive signals from GABAergic terminals. To determine whether mismatched synapse formation and elimination are regulated by the presence of GABAergic neurons, hippocampal cultures were seeded with ectopic GABAergic neurons from neonatal striatum of GAD67-EGFP mice. These cells were introduced either at the onset of synaptogenesis (after 3 DIV) or when synaptogenesis was in progress (after 6 DIV) (Studler et al., 2005). Co-cultures were kept for another 6 DIV prior to fixation and immunofluorescence staining for various synaptic proteins. During this time, a rapid growth of axons from striatal EGFP-positive GABAergic neurons establishing numerous contacts with hippocampal cells was observed. GABAergic axons from hippocampal interneurons were very few, so that the majority of VIAAT-positive terminals were derived from the ectopic GABAergic axons (not shown). Therefore, in addition to EGFP, we used secondary antibodies coupled to a green fluorochrome to stain for VIAAT for distinguishing presynaptic terminals in EGFP-positive axons in combination with two other synaptic markers.

Figure 2

Early GABAergic innervation prevents the formation of mismatched synapses in striato-hippocampal co-cultures. Striatal EGFP-positive GABAergic neurons seeded after 3 DIV and the co-cultures stained 6 days later (3+6 DIV). GABAergic neurons innervated hippocampal neurons, in which only few mismatched synapses (empty arrowheads) are evident. Each panel depicts a single confocal layer with an overlay of three color channels; the boxed area is displayed at higher magnification in color-separated images beside each panel. **A-B**: Examples of neurons



received a light (LI) and strong (SI) innervation by EGFP-positive axons, displaying gephyrin (ge; red) clusters devoid of vGluT1 staining (blue), likely representing matched synapses (filled arrowheads). **C-D**: Immunofluorescence staining for VIAAT (green) reveals presumptive presynaptic sites in the EGFP-positive axons and indicates that such gephyrin clusters devoid of vGluT1 are indeed apposed to GABAergic terminals (full arrowheads). **E**: Comparison with a hippocampal cell of the same age (9 DIV) but not innervated (NI) by GABAergic axons, which receives numerous mismatched synapses (empty arrowheads). **F**: In strongly innervated cells, some gephyrin clusters are apposed simultaneously to a VIAAT-positive terminal and a vGluT1-positive terminal, resulting in a white dot in the overlay (arrows). Scale bar for A-E = 10 μm; F = 2 μm.

Early GABAergic innervation prevents formation of mismatched synapses

We first investigated whether formation of mismatched synapses only occurs in the absence of GABAergic presynaptic input at the onset of synaptogenesis (3+6 DIV co-culture experiments). At 9 DIV, most hippocampal neurons were innervated by EGFP-positive axons, in addition to numerous vGluT1-positive terminals (Figure 2A-D). The density of GABAergic innervation was variable and we distinguished between cells receiving little innervation, i.e., one EGFP-positive axon crossing its dendrites (Figure 2A,C) and strongly innervated cells with proximal dendrites and the soma being innervated (Figure 2B,D). In both cell populations, gephyrin immunoreactive clusters on dendrites and soma were apposed to EGFP-positive axons. Lightly innervated cells had, in addition, gephyrin clusters apposed to vGluT1-positive terminals, suggesting the presence of mismatched synapses along with "proper" GABAergic synapses (Figure 2C). In contrast, strongly innervated cells were largely devoid of mismatched synapses, with most gephyrin clusters being apposed to an EGFP-positive axon (Figure 2B). Staining for VIAAT in addition to EGFP confirmed with the intense green fluorescence of terminals that gephyrin was located postsynaptically in both lightly and strongly innervated cells (Figure 2C,D). In control experiments, hippocampal neurons devoid of GABAergic input displayed numerous mismatched synapses on their dendrites and somata at 9 DIV (Figure 2E).

For quantification, only cells innervated by EGFP-positive axons were considered. Using single confocal images of cultures stained for gephyrin, VIAAT, and vGluT1, the total number of gephyrin clusters on single cells was determined. They were subdivided into four groups based on their apposition to terminals positive for VIAAT (matched), vGluT1 (mismatched), both (dual innervation), or none (extrasynaptic). Apposition was defined at

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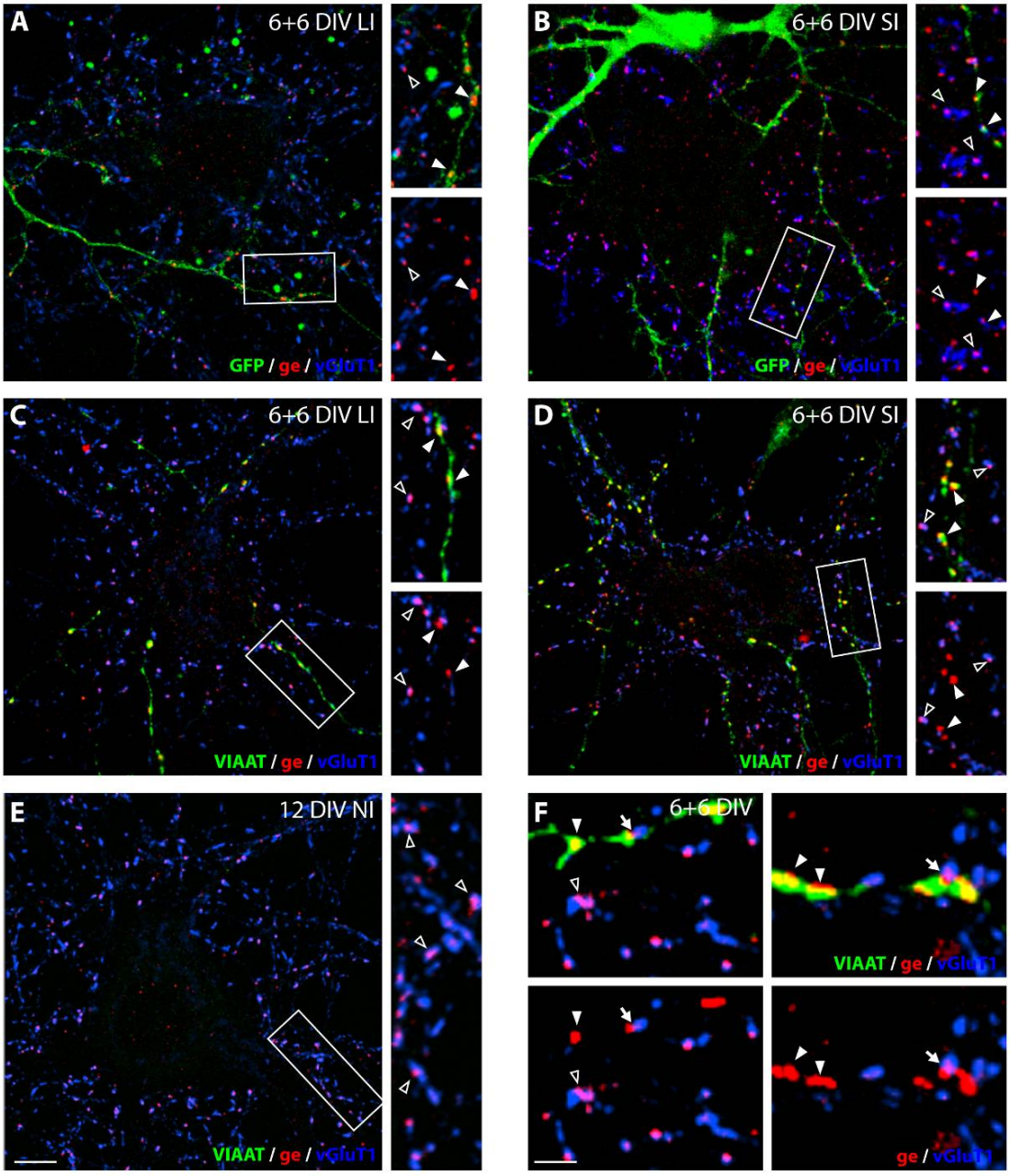
Figure 3

Mismatched synapses formed prior to GABAergic innervations persist for several days. Striatal EGFP-positive GABAergic neurons seeded after 6 DIV and the co-cultures stained 6 days later (6+6 DIV). Despite formation of GABAergic synapses (full arrowheads), mismatched gephyrin (ge) clusters persist under these conditions (empty arrowheads). Each panel depicts a single confocal layer with an overlay of three color channels; the boxed area is displayed at higher magnification in color-separated images below each panel. **A-B**: Both lightly (LI) and strongly (SI) innervated cells exhibit gephyrin clusters (red) apposed to EGFP-positive axons, denoting correctly



matched synapses (filled arrowheads), and gephyrin clusters apposed to vGluT1-positive terminals (blue), representing mismatched synapses (empty arrowheads). **C-D**: Immunofluorescence staining for VIAAT (green) confirms that gephyrin clusters devoid of vGluT1 are indeed apposed to GABAergic terminals. Mismatched synapses can be seen in their immediate vicinity. **E**: Cells of the same age (12 DIV) but devoid of GABAergic innervation (NI) exhibit a large number of mismatched gephyrin clusters (pink). **F**: Similarly to the 3+6 cultures, a subset of gephyrin clusters is apposed to both VIAAT-positive terminals and vGluT1-positive terminals (arrows). Scale bar for A-E = 10 μm; F = 2 μm.

high magnification by direct contact or overlay for each marker. In 3+6 DIV cultures (Figure 4A), 26 ± 18 gephyrin clusters (mean ± SD) were apposed to a GABAergic terminal (48% of the total gephyrin clusters), 14 ± 11 (27%) were mismatched, 4 ± 3 (6%) were dually innervated, and 11 ± 9 (19%) were located extrasynaptically. Analysis of cluster size revealed that matched gephyrin clusters were significantly larger than mismatched clusters ($p<0.001$, Kolmogorov-Smirnov; Figure 4B).

While outgrowth of GABAergic terminals during the early phase of synaptogenesis largely prevented formation of mismatched synapses, a sizeable fraction of gephyrin clusters were apparently dually innervated (Figure 2F) in both, lightly and strongly innervated hippocampal neurons (see Figure 4 for quantification). Since gephyrin clusters are located postsynaptically in hippocampal cell dendrites, such dually innervated clusters gave possible hints about the mechanism of synapse formation. Concerted interactions between a glutamatergic and a GABAergic axon for formation of a synaptic site appear rather unlikely. Therefore, dually innervated gephyrin clusters suggest that postsynaptic sites are inductive for synapse formation by attracting both types of terminals.

Delayed GABAergic innervation does not induce rapid elimination of mismatched synapses

Next, we examined whether mismatched synapses represent transient contacts rapidly eliminated upon GABAergic innervation using co-cultures seeded with ectopic GABAergic cells at 6 DIV and maintained *in vitro* for another 6 days prior to fixation and staining (6+6 DIV). At 6 DIV, synaptogenesis already led to the formation of mismatched synapses, as verified in control cultures (not shown). Analysis of 6+6 DIV cultures again revealed that ectopic GABAergic cells successfully contacted their target cells, providing either a light (Figure 3A,C) or a strong innervation (Figure 3B,D). Additional staining for VIAAT confirmed that ectopic GABAergic terminals were apposed to gephyrin clusters, forming presumptive GABAergic synapses (Figure 3C-D). The total number of gephyrin clusters per cell increased significantly between 3+6 and 6+6 cultures ($p<0.001$, $n=30$, unpaired *t*-test). Moreover, unlike 6+3 DIV cultures, both populations of hippocampal cells carried numerous mismatched synapses often located in close vicinity to the properly matched

synaptic contacts (Figure 3C-D). Examination of control neurons receiving no GABAergic innervation

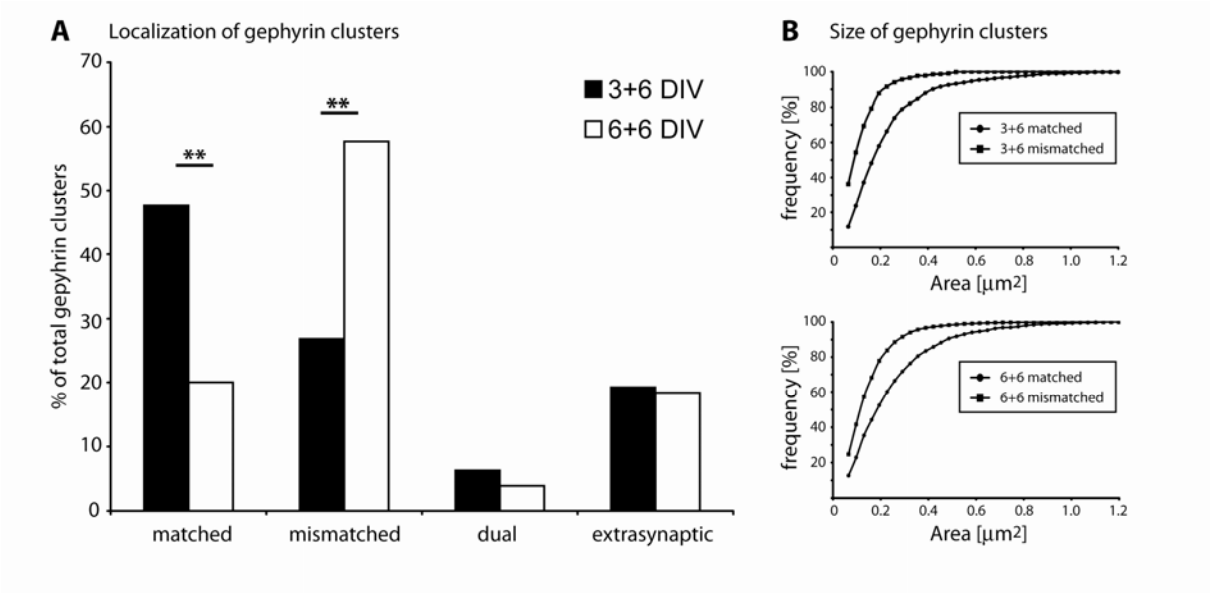


Figure 4

Effect of GABAergic innervation on the proportion and size of matched and mismatched gephyrin clusters in 3+6 DIV and 6+6 DIV co-cultures. **A**: Relative distribution of gephyrin clusters apposed to VIAAT-positive terminals (matched), vGluT1-positive terminals (mismatched), both terminal types (dual), and neither (extrasynaptic). The total number of gephyrin clusters per cell was 56 ± 26 in 3+6 cultures and 135 ± 29 in 6+6 DIV cultures. The proportion of matched clusters was significantly higher in 3+6 DIV than in 6+6 cultures, whereas mismatched clusters were more numerous at 6+6 DIV than 3+6 DIV (**, $p < 0.001$, post-hoc *t*-test). The fraction of dually innervated and extrasynaptic gephyrin clusters was not different between the two time-points. **B**: Cumulative probability plots of the size of gephyrin clusters forming matched versus mismatched synapses in 3+6 and 6+6 cultures revealing a significant enlargement of matched clusters compared to mismatched clusters ($p < 0.001$, Kolmogorov-Smirnov).

after 12 DIV corroborated the steady formation of mismatched synapses uniformly distributed on dendrites during this time in culture (Figure 3E). Statistically, an ANOVA with repeated measures on the same subjects (Figure 4A) confirmed that the distribution of the four types of gephyrin clusters was significantly different in 3+6 and 6+6 cultures ($F_{3,174}$=32.95, n=30 cells per culture condition, $p<0.001$). The fraction of mismatched clusters was significantly higher in 6+6 DIV compared to 3+6 DIV cultures (58% versus 27%, $p<0.001$, post-hoc *t*-test; Figure 4A); the proportion of matched clusters was lower at 6+6 DIV than at 3+6 DIV (20% and 48%, respectively), and the other two groups were

unchanged. Again, the size of matched gephyrin clusters was significantly larger than that of mismatched clusters ($p<0.001$, Kolmogorov-Smirnov; Figure 4B). Altogether, these results

indicate that GABAergic presynaptic terminals formed numerous synapses between 6 and 12 DIV without affecting mismatched synapses formed prior to their arrival.

As noted above, the fraction of "dually-innervated" gephyrin clusters was similar in 3+6 DIV and 6+6 DIV co-cultures (6% versus 4% of total gephyrin clusters, respectively; Figure 4). Since numerous mismatched synapses were already formed at the time of seeding of GABAergic cells, this observation suggests that GABAergic axons innervating hippocampal cells are still "attracted" to gephyrin clusters, even when they are already contacted by a glutamatergic terminal, providing further support to the hypothesis that GABAergic PSDs, marked by gephyrin clusters, are inductive of synapse formation *in vitro*.

1.5. Discussion

In this study, we show that (1) NL2 clusters are associated with $GABA_A$ receptors and gephyrin at mismatched synapses formed by cultured hippocampal neurons when presynaptic GABAergic input is sparse; (2) GABAergic input made available at the onset of synaptogenesis prevents the formation of mismatched synapses; (3) mismatched synapses that are already established when GABAergic innervation is provided are not eliminated by replacement of the presynaptic terminal but persist for days; (4) a fraction of postsynaptic clusters are contacted by both, a GABAergic and a glutamatergic terminal, suggesting a postsynaptic signaling mechanism triggering synapse formation onto a pre-existing PSD. Altogether, these findings suggest a model whereby nascent or pre-formed postsynaptic protein clusters containing NL2 might initiate GABAergic synapse formation by stabilizing contacts between axon terminals and dendrites, and/or activating trans-cellular signaling required for recruitment of synaptic molecules and differentiation of the synaptic apparatus. At least the early steps of this process appear to occur independently of GABA-mediated transmission.

Methodological considerations

For having a better control over the developmental stage of neurons at the moment of GABAergic innervation, we exploited the delayed development of GABAergic interneurons in low density hippocampal primary cultures and introduced ectopic GABAergic cells before the onset of synaptogenesis or after formation of mismatched synapses. In low density cultures, the first α2 subunit and gephyrin clusters are detectable around 4 DIV and their density increases rapidly thereafter (Studler et al., 2005). Therefore, ectopic GABAergic cells seeded onto hippocampal neurons at 3 DIV participate to the onset of synaptogenesis whereas those seeded at 6 DIV innervate cells carrying mismatched synapses. The development of striatal GABAergic cells was similar regardless of the maturation of their postsynaptic targets and they rapidly formed ectopic synapses onto pyramidal cells. The precise apposition of pre- and postsynaptic markers on the dendrites of cultured neurons strongly argues that they represent synaptic sites, and, as shown previously, the α2 subunit staining can be considered a surrogate for $GABA_A$ receptor

complexes containing also β and γ subunit variants (Brunig et al., 2002b; Studler et al., 2002; Studler et al., 2005).

To avoid confounding effects from hippocampal GABAergic cells, the quantitative analysis was restricted to neurons receiving GABAergic synapses only from ectopic cells. The total number of gephyrin clusters was significantly different at 3+6 and 6+6 DIV (Figure 4A), reflecting the fact that synaptogenesis is rapid between 9 and 12 DIV and that the presence of ectopic neurons did not affect hippocampal cell growth and maturation. It is important to note that, *in vivo*, hippocampal GABAergic synapses form before glutamatergic synapses (Gozlan and Ben-Ari, 2003); this sequence has been speculated to prevent over-excitability of hippocampal circuits by ensuring shunting inhibition to counteract the excitatory drive of glutamate receptors (reviewed in Ben-Ari, 2002). GABAergic transmission has also been proposed to exert trophic functions during development, owing to the depolarizing action of $GABA_A$ receptors in immature neurons. However, in the cell culture model used here, no obvious difference in neuronal maturation or morphology was observed in cultures receiving early or delayed GABAergic input.

NL2 is present at mismatched synapses

Mismatched synapses formed on hippocampal neurons are well documented (Rao et al., 2000; Brunig et al., 2002b; Studler et al., 2005) and have been shown to contain gephyrin and $GABA_A$ receptors, but not proteins of the dystrophin-associated complex (Brunig et al., 2002b; Levi et al., 2002). The presence of NL2 in mismatched synapses, demonstrated here, adds to the list of postsynaptic proteins that are clustered in a cell autonomous way independently of the neurotransmitter present in axon terminals. Furthermore, several lines of evidence support the view that NL2 clustering might be an early process in the formation of a GABAergic PSD: 1) Up- or down-regulation of NL2 expression leads to an increase or decrease of GABAergic synapses, respectively, as shown *in vitro* (Chih et al., 2005) and *in vivo* (Chubykin et al., 2007). 2) Differential binding of NL2 to α- and β-neurexin splice variants is a key determinant of GABAergic versus glutamatergic synapse formation (Kang et al., 2008); 3) In cells devoid of functional $GABA_A$ receptors, such as Purkinje cells of $\alpha_1^{0/0}$ mice, NL2 is absent from mismatched GABAergic synapses formed

on spines but is present in symmetric synapses on dendrites, suggesting a role for the formation of symmetric synapses (Patrizi et al., 2008). 4) In the present work, NL2 and gephyrin were extensively co-localized at synaptic sites, whereas NL2 clusters without gephyrin were mainly extrasynaptic. Therefore, clustering of NL2 might occur without gephyrin at the onset of PSD formation. To confirm the role of NL2 for synaptic differentiation, it would be of interest to determine ultrastructurally whether mismatched synapses formed in hippocampal neurons are symmetric synapses.

In contrast to NL2, postsynaptic clustering of gephyrin is abolished in the absence of $GABA_A$ receptors (Kralic et al., 2006; Patrizi et al., 2008; Peden et al., 2008). Furthermore, gephyrin clustering at GABAergic synapses depends on interaction with the brain specific GDP/GTP exchange factor collybistin (Harvey et al., 2004; Papadopoulos et al., 2007; Papadopoulos et al., 2008). The formation of a GABAergic PSD therefore requires proper targeting and clustering of $GABA_A$ receptors, which, in turn might induce gephyrin clusters in a collybistin-dependent process. It is not known how NL2 interacts with GABA receptors or with gephyrin, but recombinant expression of NL2 along with $GABA_A$ receptors in HEK293 cells (which express endogenous gephyrin) leads to formation of functional synapses (Dong et al., 2007). In neurons, the roles of presynaptic terminals for GABAergic synaptogenesis might be to stabilize NL2 via interactions with neurexins (reviewed in Huang and Scheiffele, 2008) and to provide signaling molecules regulating collybistin activity and/or recruitment of postsynaptic proteins contributing to PSD formation.

GABAergic postsynaptic proteins influence GABAergic synapse formation

Interestingly, knockdown of gephyrin or $GABA_A$ receptors significantly reduces the density of presynaptic GABAergic innervation onto cultured neurons (Levi et al., 2004; Li et al., 2005; Fang et al., 2006; Yu et al., 2007). We have also shown that *in vitro* overexpression of a gephyrin mutant protein that forms supernumerary postsynaptic clusters in hippocampal neurons increases the number of GABAergic synapses at the expense of glutamatergic synapses (Lardi-Studler et al., 2007). Here, the presence of a substantial fraction of gephyrin clusters apposed to both a VIAAT- and a vGluT1-positive terminal provides further evidence that postsynaptic structures containing gephyrin clusters might facilitate synapse formation. Gephyrin is a cytoplasmic protein unlikely to be directly

involved in this process; rather, as noted above, gephyrin clusters are closely associated with NL2, which could interact with presynaptic neurexins; alternatively, preformed PSDs might present other transmembrane molecules that attract or stabilize presynaptic terminals; finally, when contacted by a presynaptic terminal, pre-formed PSDs might mediate signaling cascades leading to the rapid stabilization of the initial contact.

The significant size difference of properly matched gephyrin clusters compared to mismatched gephyrin clusters (Figure 4B) indicates that functional neurotransmission leads to an enlargement of the PSD, probably due to accumulation of postsynaptic proteins, which could be essential for the long-term stability of synaptic contacts. This observation is in line with the deficit in GABAergic innervation and function reported *in vivo* when GABA synthesis is reduced (Chattopadhyaya et al., 2007). Furthermore, it suggests that neurotransmitter-mediated signals might regulate the size of PSD through post-translational modification of pre- or postsynaptic scaffolding proteins.

Mismatched synapses are not eliminated rapidly

Mismatched synapses formed before the arrival of GABAergic innervation are not eliminated rapidly but persist for several days *in vitro*. It appears unlikely that ectopic GABAergic axon terminals replace glutamatergic axons in mismatched synapses. Therefore, despite being non-functional, mismatched synapses are stable structures, whose disappearance might occur at later stages of neuronal maturation. As a correlate, functional synaptic transmission is not required for synapse maintenance, at least on a time-scale of days *in vitro*. This conclusion is confirmed by the long-term stability of GABAergic synapses formed by cerebellar basket cells onto Purkinje cells of $\alpha_1^{0/0}$ mice, which persist up to adulthood despite the lack of $GABA_A$ receptors (Fritschy et al., 2006). It is noteworthy that GABAergic input at early stages of synaptogenesis in hippocampal cultures largely prevents the formation of mismatched synapses, thereby revealing a competitive advantage of GABAergic terminals over glutamatergic terminals and/or the existence of inhibitory mechanisms preventing mismatched synapse formation in the vicinity of GABAergic synapses, as suggested by Christie et al., (2002). This inhibition might be due to depletion of synaptogenic molecule(s), such as NL2, upon formation of GABAergic synapses.

Conclusions

The present results suggest a model of GABAergic synaptogenesis, whereby nascent or pre-formed GABAergic postsynaptic protein clusters containing NL2 attract axonal innervation (Huang and Scheiffele, 2008). Synaptic differentiation might be initiated upon contact of axon terminals at these sites, presumably through activation of trans-synaptic signaling pathways, leading to further recruitment of synaptic proteins. The presence of extrasynaptic NL2 clusters not colocalized with gephyrin in cultured neurons suggests a role of NL2 for initiating synapse formation. Differentiation of the PSD, denoted by clustering of $GABA_A$ receptor and gephyrin, appears to be largely independent of the neurotransmitter involved, thereby explaining the presence of NL2, gephyrin, and the $\alpha 2$ subunit in both matched and mismatched synapses. However, GABAergic terminals are at a competitive advantage over glutamatergic terminals in recruiting GABAergic postsynaptic molecules, and functional neurotransmission leads to the formation of larger postsynaptic proteins clusters. Therefore, GABAergic transmission might contribute to regulate the rate of synaptogenesis and the development of GABAergic synapses.

1.6. Acknowledgments

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2. Study II: Assembly of $GABA_A$ receptors and neuroligin2 in developing GABAergic synapses on cerebellar Purkinje cells is gephyrin-independent in collybistin-knockout mice

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2.1. Abstract

The small Rho-like GTPase collybistin is specifically expressed in the brain. It is known to directly interact with the GABAergic synaptic scaffolding protein gephyrin and affects its synaptic clustering *in vitro*. Unexpectedly, *in vivo*, collybistin-knockout mice showed impaired gephyrin clustering only in a region-specific manner at GABAergic synapses, suggesting an unsuspected heterogeneity in the molecular organization at GABAergic postsynaptic sites. To clarify the role of collybistin in postsynaptic organization, it will be necessary to determine how the lack of collybistin impairs gephyrin clustering in specific synapses and whether additional postsynaptic molecules contribute to this effect. In particular, the role of neuroligin2, a synaptogenic transmembrane protein selectively enriched in GABAergic synapses needs to be evaluated in collybistin-knockout mice.

Here, using immunohistochemistry, we analyzed GABAergic synapse formation during postnatal development in cerebellar Purkinje cells of collybistin-knockout mice. We investigated whether absence of gephyrin clusters is constitutive in collybistin-knockout animals and impairs or delays clustering of the $GABA_A$ receptor and neuroligin2 during GABAergic synaptogenesis. We observed that gephyrin is not clustered at synaptic sites at any developmental stage and that formation of GABAergic synapses and clustering of $GABA_A$ receptors and neuroligin2 in Purkinje cells do not require collybistin-dependent gephyrin clustering.

2.2. Introduction

Collybistin is a brain-specific guanine nucleotide exchange factor of the Dbl-family acting on small Rho-like GTPases. The protein is composed of an N-terminal SH3-, a Dbl-homology (DH), and a pleckstrin-homology (PH) domain. The DH domain incorporates the GDP/GTP exchange unit whereas the PH domain is required for collybistin activation upon binding to phosphatidylinositols in the plasma membrane. Several splice variants of collybistin have been identified differing in three variants of their C-terminal sequences (collybistin1-3) as well as the presence or absence of the SH3 domain, whereas the vast majority of collybistin isoforms encode for the collybistin2 and collybistin3 variants bearing the SH3 domain (Kins et al., 2000; Harvey et al., 2004). The human homologue of collybistin (hPEM-2) specifically activates Cdc42 (Reid et al., 1999), which in turn is known to regulate the organization of actin filaments (Hall, 1998). Collybistin has been identified as binding partner of gephyrin inducing its submembrane clustering upon co-expression in non-neuronal cells (Kins et al., 2000; Grosskreutz et al., 2001). In primary hippocampal cultures, overexpression of collybistin mutants lacking either the PH domain or the DH domain, which also contains the gephyrin binding site, exert dominant-negative effects on endogenous collybistin function and reduce gephyrin clustering at postsynaptic sites, resulting in impaired GABAergic function (Harvey et al., 2004). The crystal structure of Cdc42 in complex with collybistin2 showed that both domains are involved in proper collybistin-function and in the same study, biochemical analysis revealed that gephyrin bindings inhibits collybistin activity (Xiang et al., 2006). Therefore, while collybistin was considered crucial for clustering of gephyrin and other synaptic proteins at GABAergic and glycinergic postsynaptic sites, its precise function remains elusive.

Unexpectedly, analysis of collybistin-deficient (collybistin-KO) mice revealed that gephyrin clustering was not affected at glycinergic synapses, while being impaired in a region-specific manner at GABAergic synapses (Papadopoulos et al., 2007). Notably, a profound loss of gephyrin and $GABA_A$ receptor clusters was observed in the hippocampus and amygdala, affecting synaptic plasticity, whereas gephyrin clusters, but not $GABA_A$ receptors, were absent in the molecular layer of the cerebellum (Papadopoulos et al., 2007). These results revealed an unsuspected heterogeneity in the molecular organization

of inhibitory postsynaptic sites and suggested that alternative mechanisms of gephyrin and $GABA_A$ receptor clustering exist in specific subsets of GABAergic synapses.

To clarify the role of collybistin in the functional organization of postsynaptic sites, it will be necessary to determine how the lack of collybistin impairs gephyrin clustering in specific synapses and whether additional postsynaptic molecules contribute to this effect. In particular, the role of neuroligin2 (NL2), a NL isoform selectively enriched in GABAergic synapses (Varoqueaux et al., 2004), needs to be evaluated in collybistin-KO mice.

NLs display synaptogenic activity and thus might provide alternative pathways for recruiting proteins at postsynaptic sites. *In vitro*, the interaction of NLs and their presynaptic binding partners α- and β-neurexins are sufficient to induce clustering of synaptic proteins and morphological differentiation of synapses (Biederer and Stagi, 2008). *In vivo*, NLs are dispensable for synapse formation but control the balance of excitatory and inhibitory synapses during brain maturation (Chih et al., 2005; Varoqueaux et al., 2006; Sudhof, 2008). NL1 and NL2 are selectively localized at glutamatergic and GABAergic postsynaptic densities, respectively (Song et al., 1999; Varoqueaux et al., 2004); their relative expression level determines the number of each type of synapses formed in developing neurons (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). Therefore, NL2 can be considered as a major candidate protein regulating the formation of GABAergic synapses. Whether NL2 clustering is impaired in collybistin-KO mice is not known.

Here, we analyzed whether absence of collybistin leads to a constitutive impairment of gephyrin and/or NL2 clustering in GABAergic synapses on Purkinje cells during development, and whether such impairment might delay the formation of GABAergic synapses. The cerebellar cortex was used for this study because synaptogenesis occurs postnatally and because GABAergic synapses in Purkinje cells contain homogeneous $GABA_A$ receptors composed of the $\alpha1\beta2/3\gamma2$ subunits (Laurie et al., 1992a; Fritschy et al., 2006). Using immunohistochemistry, we compared the distribution of pre- and postsynaptic markers in WT and mutant littermates at postnatal day 7 (P7), P10 and P20, corresponding to the onset, peak, and termination of GABAergic synaptogenesis. We observed that gephyrin is not clustered at synaptic sites at any developmental stage and that formation of GABAergic synapses and clustering of $GABA_A$ receptors and NL2 in Purkinje cells do not require collybistin-dependent gephyrin aggregation.

2.3. Material and Methods

Animals

All experiments were performed on WT and collybistin-KO mice generated on a C57BL/6J background at the Max-Planck-Institute for Brain Research in Frankfurt, Germany (Papadopoulos et al., 2007) and obtained from heterozygous (collybistin$^{+/0}$) female mice crossed with WT C57BL/6J male animals. The mouse collybistin gene (Arhgef9) is located on the X-chromosome and therefore only male offspring which have either the WT or KO allele were used. Mice were genotyped by PCR analysis of tail DNA. All experiments were performed according to the international guidelines on animal care and use and were approved by the cantonal veterinary office of Zurich.

Immunohistochemistry

For immunohistochemical detection of different pre- and postsynaptic proteins the following antibodies were used: guinea pig antisera against $GABA_A$ receptor subunits α1 and γ2 (1:10 000 and 1:2000, respectively; raised in house and characterized in Benke et al., (1991a); Benke et al., (1991b); Fritschy and Mohler, (1995), mouse anti-gephyrin mAb7a (1:400; Synaptic Systems, Göttingen, Germany), rabbit anti-NL2 (1:2000; characterized in Varoqueaux et al., (2004) and affinity purified rabbit antiserum against VIAAT (1:1000; Synaptic Systems).

The postsynaptic co-localization of $GABA_A$ receptor subunits α1 or γ2 with NL2 and gephyrin was analyzed in WT and collybistin-KO mice using triple immunofluorescence staining in fresh-frozen tissue sections. Mice (P7, P10 and P20; n=3 per age and genotype) were anesthetized with ether and decapitated. The brain was extracted rapidly and frozen with powdered dry ice. Parasagittal sections through the vermis were cut at 14 μm with a cryostat, mounted onto gelatin-coated glass slides, air dried at room temperature and stored at –20°C. They were then thawed at room temperature again and fixed in methanol at –20°C for 1-2 min. After 2 washes in PBS, the sections were incubated overnight at 4°C with a mixture of primary antibodies diluted in PBS containing 4% normal goat serum. Sections were washed extensively in PBS and incubated for 30

min at room temperature with the corresponding secondary antibodies conjugated to Cy3 (1:500, Jackson Immunoresearch, West Grove, PA, USA), Cy5 (1:200, Jackson Immunoresearch) or Alexa488 (1:1000, Molecular Probes, Eugene, OR, USA). Sections were washed again with PBS and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA, USA).

For co-visualization of presynaptic terminals and gephyrin clusters in young WT and collybistin-KO mice (P7 and P10; n=2-3 per age and genotype), weakly fixed tissue sections were prepared from living slices as described in (Schneider Gasser et al., 2006). Mice were anesthetized and decapitated as above and the brain rapidly removed into oxygenated "ice-cold" artificial cerebrospinal fluid (aCSF). 300 μm thick slices were cut with a vibratome and incubated for 30 min in aCSF at 34°C. They were then fixed in 4% paraformaldehyde (EMS, Hatfield, PA, USA), pH 7.4 in 0.15 M phosphate buffer for 20 min, extensively washed and incubated overnight in 30% sucrose in PBS for cryoprotection. Parasagittal sections (16 μm) were cut from frozen slices, mounted onto SUPER FROST GOLD glass slides (Menzel-Gläser, Braunschweig, Germany), air dried at room temperature, and stored at –20°C. Triple immunofluorescence staining was performed as described above.

In older mice (P20), synaptic markers can be detected optimally using pepsin-treatment of perfusion fixed tissue. For these experiments, mice were anesthetized with Nembutal (50 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde and 0.2% picric acid in 0.15 M phosphate buffer pH 7.4. The brains were immediately removed and postfixed for another 3 h in the same fixative. Brains were then cryoprotected with 30% sucrose in PBS overnight, and frozen. Parasagittal sections through the vermis were cut at 40 μm with a sliding microtome and stored in an antifreeze solution containing 15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4 at -20°C. For antigen retrieval, free floating sections were incubated in 0.15 mg/ml pepsin in 0.2 M HCl for 10 min at 37°C (Watanabe et al., 1998). After extensive washing, the sections were processed for triple immunofluorescence staining in PBS containing 4% normal goat serum and 0.1% Triton X-100 as described above.

Image analysis:

Immunofluorescence staining was visualized by confocal laser scanning microscopy (Zeiss LSM-510 Meta; Jena, Germany) using a 100x Plan-Apochromat objective (N.A. 1.4). The pinhole was set to 1 Airy unit for each channel and color channels were acquired sequentially. The acquisition settings were adjusted to cover the whole dynamic range of the photomultipliers. For display, images were processed with the image-analysis program Imaris (Bitplane; Zurich, Switzerland) or ImageJ (NIH, Bethesda, MD, USA). Images from three consecutive confocal sections representing a 0.9 μm stack of all channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. An anisotropic diffusion filter for edge preserving was used for images displaying α1, γ2 or VIAAT staining.

2.4. Results

Postsynaptic proteins cluster properly during early synaptic development

The absence of collybistin leads to a loss of gephyrin clusters in adult cerebellar Purkinje cells (Papadopoulos et al., 2007). To investigate whether clustering of gephyrin (and other postsynaptic proteins) is constitutively impaired or whether gephyrin clusters are unstable and gradually disappear from mature GABAergic synapses, we investigated the distribution of gephyrin, $GABA_A$ receptor subunits α1 and γ2, and NL2 in Purkinje cells during postnatal development. At P7, when the first GABAergic synapses are established by basket cell axon terminals on Purkinje cell somata and proximal dendrites, gephyrin immunoreactivity in WT mice was present in clusters and co-localized with the $GABA_A$ receptor γ2 and α1 subunits, as well as with NL2 (Figure 1A, C). In collybistin-KO mice, gephyrin was not detectable in the molecular layer. Nevertheless, the γ2 and α1 subunit formed clusters co-localized with NL2 at presumptive GABAergic postsynaptic sites (Figure 1B, D), reflecting proper targeting and aggregation of GABAergic postsynaptic proteins at early steps of GABAergic synaptogenesis despite the absence of gephyrin clusters. Up to P20, numerous GABAergic synapses from stellate cells are formed on the developing dendritic arbor of Purkinje cells. As seen both at P10 and P120, gephyrin clustering was abrogated in the

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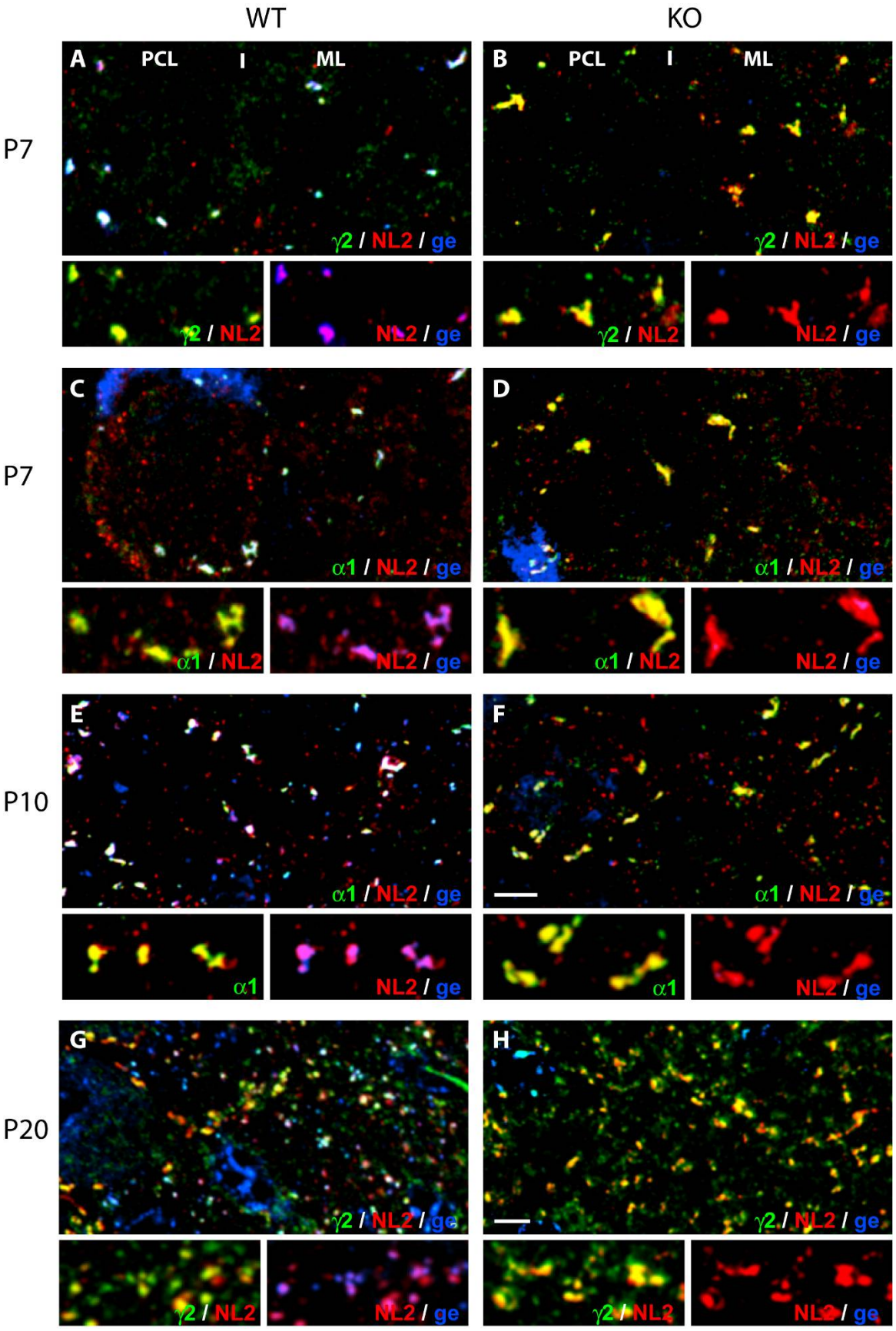
Figure 1

Unaltered clustering of postsynaptic proteins during early phases of GABAergic synaptogenesis in Purkinje cells of collybistin-KO mice, as visualized by immunofluorescence staining and confocal laser scanning microscopy. **A-D**: At P7, initial synapses on Purkinje cell somata and proximal dendrites are observed based on co-clustering of the α1 or γ2 subunit (green) with NL2 (red) and gephyrin (blue). In collybistin-KO mice, clusters are formed at this age, but they are devoid of gephyrin staining (B, D). At later developmental stages, P10 (**E, F**) P20 (**G, H**), gephyrin clusters are likewise constitutively absent in sections from collybistin-KO mice (**F, H**). Nevertheless, clusters double-labeled for NL2 and $GABA_A$ receptor subunits were found on both genotypes and their density increased markedly between P10 (**E, F**) and P20 (**G, H**), suggesting a normal development of GABAergic synapses in collybistin-KO mice. Note the disappearance of gephyrin clusters on the soma of Purkinje cells between P10 and P20 in WT mice (**C, E**). Abbreviations: PCL, Purkinje cell layer; ML, molecular layer. Scale bars = 5 μm (A-F and G-H).


WT
KO
P7
P7
P10
P20
A
PCL
I
ML
γ2 / NL2 / ge
γ2 / NL2
NL2 / ge
B
C
α1 / NL2 / ge
α1 / NL2
D
E
α1
F
G
H

molecular layer of collybistin-KO mice, suggesting inability of gephyrin to form aggregates in Purkinje cells (and cerebellar interneurons) in the absence of collybistin (Figure 1F). In WT mice, the density of gephyrin clusters in the molecular layer increased steadily between P10 and P20, whereas gephyrin clusters disappeared from the soma of Purkinje cells, as reported previously (Viltono et al., 2008). Nevertheless, in WT and in collybistin-KO mice, α1-subunit is found in clusters co-localizing with NL2 on the soma and dendrites of Purkinje cells to a similar extent in both, WT and collybistin-KO animals (Figure 1E-H). We therefore conclude that postsynaptic clustering of GABAergic proteins does not depend on gephyrin aggregation at any stage of GABAergic synaptogenesis.

Presynaptic terminals form at proper sites in collybistin-KO animals

To determine whether the maturation of GABAergic synapses might be affected in mutant mice and to demonstrate that clusters of α1/γ2 subunit and NL2 immunoreactivity are postsynaptic despite impairment of gephyrin clustering, we examined the distribution of presynaptic terminals visualized by VIAAT-immunofluorescence at the same developmental stages as above. In WT mice, we confirm previous observations that VIAAT puncta at P7 are located mainly on Purkinje cell somata and at proximal dendrites (Ango et al., 2004), apposed to postsynaptic clusters of $GABA_A$ receptor α1-subunit and gephyrin in WT animals (Figure 2A). In collybistin-KO mice, α1 subunit-positive clusters were likewise found apposed to VIAAT-positive puncta to a similar extent as in WT animals, pointing to unaffected formation of presynaptic terminals at appropriate sites (Figure 2B). The same conclusion was reached with co-staining for NL2 and the α1 subunit, found apposed to

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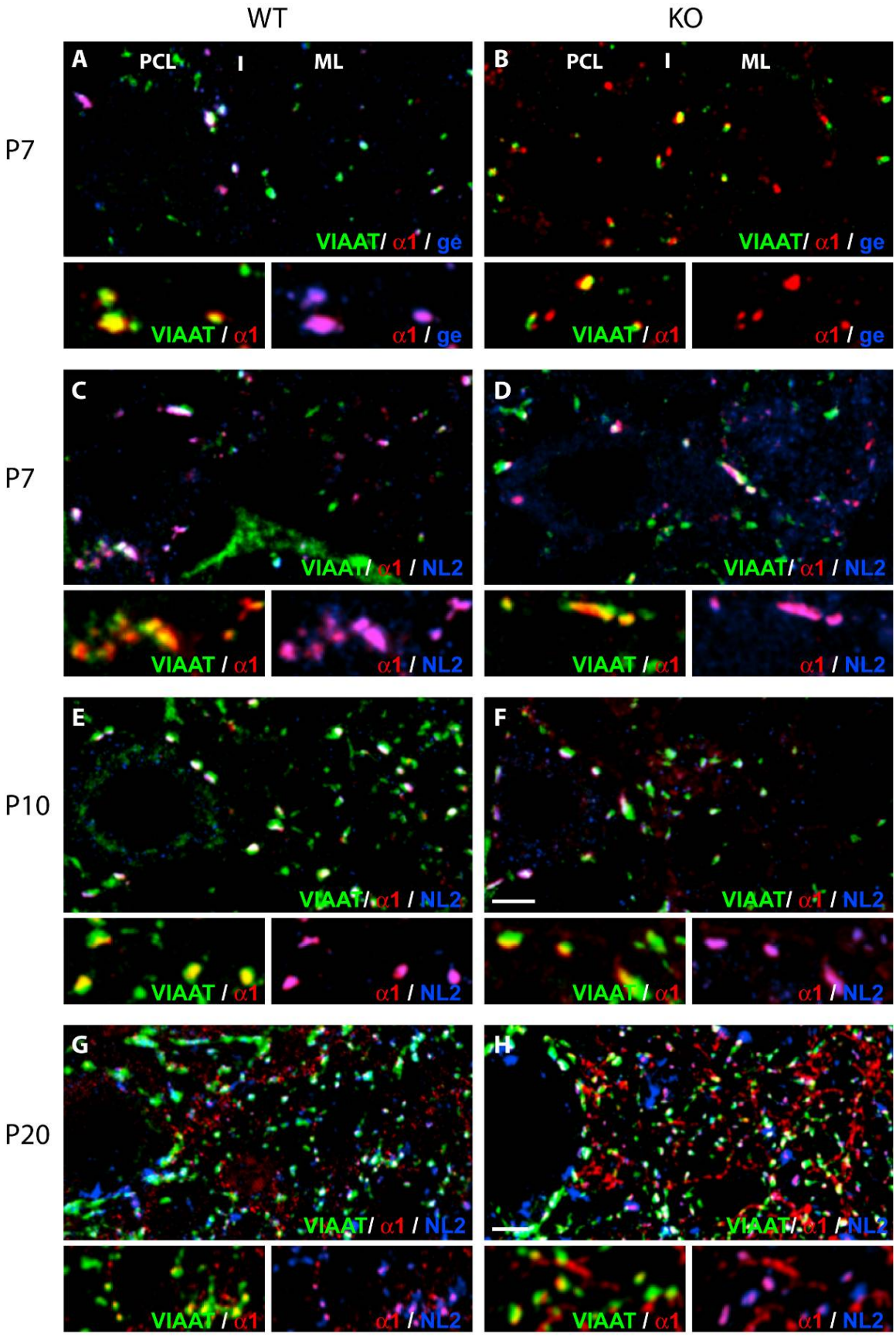
Figure 2

Proper development of presynaptic terminals apposed to postsynaptic protein clusters in collybistin-KO mice. At P7, postsynaptic clusters of $GABA_A$ receptor α1 subunit (red) and gephyrin (blue) are co-localized and apposed to VIAAT-positive terminals (green) of GABAergic presynaptic terminals (**A**). In P7 collybistin-KO mice, gephyrin is undetectable but α1 subunit-positive clusters are found apposed to VIAAT-positive terminals (**B**). **C-H**: At P10 and P20, NL2-positive staining (blue) is found co-clustered with the α1 subunit facing GABAergic terminals in WT and collybistin-KO mice, suggesting correct targeting of postsynaptic proteins at all phases of GABAergic synaptogenesis. The age-related increase of synapse density appears similar in both genotypes. Scale bars = 5 μm (A-F and G-H).


WT
KO
P7
P7
P10
P20
A
PCL
I
ML
VIAAT/ α1 / ge
VIAAT / α1
α1 / ge
B
C
VIAAT/ α1 / NL2
α1 / NL2
D
E
F
G
H

VIAAT-positive terminals in both genotypes (Figure 2C,D). The proper development of presynaptic terminals and their association with postsynaptic proteins in collybistin-KO mice was seen also at P10 and P20 (Figure 2 E-H). The increase in numerical density of pre- and postsynaptic clusters was similar in both genotypes, indicating that lack of gephyrin clustering does not affect formation of GABAergic synapses in developing Purkinje cells of collybistin-KO mice.

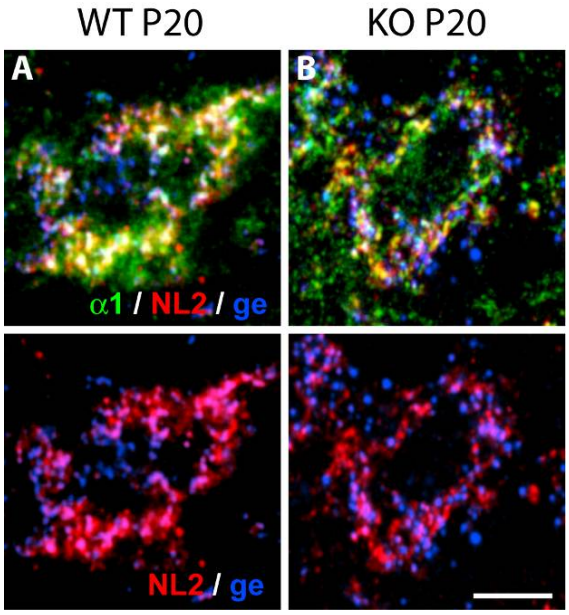


Figure 3

Gephyrin clustering around granule cell glomeruli is not impaired in collybistin-KO mice. A: In P20 WT animals, clusters of gephyrin (blue) as well as a more diffuse staining for NL2 (red) and the $GABA_A$ receptor subunit α1 (green) are co-localized of granule cell dendrites surrounding a synaptic glomerulus. **B**: In collybistin-KO mice, a similar staining pattern as in WT can be observed, suggesting that clustering of gephyrin and other postsynaptic proteins are not affected in granule cells. Scale bar = 5 μm.

Gephyrin clustering in the granule cell layer is normal in collybistin-KO animals

The loss of gephyrin clustering in the cerebellum of collybistin-KO mice occurs specifically in GABAergic synapses formed in the molecular layer on Purkinje cells and interneurons. In the granule cell layer, gephyrin clusters in GABAergic synapses formed on granule cell dendrites around glomeruli, remain unaffected, as seen when comparing WT (Figure 3A) and collybistin-KO mice (Figure 3B) at P20. Co-staining for NL2 and the α1 subunit revealed co-localization of all three GABAergic postsynaptic proteins in both genotypes. This observation suggests that clustering of gephyrin in granule cells is independent of the presence of collybistin, pointing to a different mechanism as in Purkinje cells.

2.5. Discussion

The present results confirm the neuron-specific impairment of gephyrin clustering in the cerebellum of collybistin-KO mice and demonstrate that formation of GABAergic synapses and postsynaptic clustering of $GABA_A$ receptors and NL2 are not affected by the absence of gephyrin clusters. We also show that the lack of gephyrin clustering is constitutive in collybistin-KO mice and does not occur as a consequence of decreased stability of gephyrin aggregates. Altogether, these results indicate collybistin-dependent clustering of gephyrin is dispensable for targeting and accumulation of $GABA_A$ receptors NL2 at GABAergic postsynaptic sites.

The mechanisms underlying the differential loss of collybistin-dependent gephyrin clustering in different cell types is not known. While compensation by a homologous guanine nucleotide exchange factor (Thiesen et al., 2000) represents an obvious possibility, there is no direct evidence so far that such substitution actually takes place in collybistin-KO mice. A second possibility is that gephyrin clustering is regulated differently in various types of synapses; in particular, its preservation in glycinergic synapses (Papadopoulos et al., 2007), while unexpected from in vitro studies of collybistin-gephyrin (Kins et al., 2000; Grosskreutz et al., 2001; Harvey et al., 2004), strongly argues for a collybistin-independent clustering mechanism, possibly involving interactions with accessory motor proteins, such as Dlc1/2 (Fuhrmann et al., 2002) and direct high affinity binding of gephyrin to the glycine receptor β subunit. Since inhibitory synapses around granule cell layer glomeruli also contain a glycinergic component (Sassoe-Pognetto et al., 2000), such a scenario would explain the preservation of gephyrin clusters in the cerebellar granule cell layer. Heterogeneity of GABAergic postsynaptic densities is also suggested by the differential loss of $GABA_A$ receptor clusters among brain regions of collybistin-KO mice (Papadopoulos et al., 2007). Furthermore, in a preliminary analysis of the thalamic ventrobasal complex, a region devoid of glycinergic synapses, we observed a preservation of gephyrin clusters in collybistin-KO mice (unpublished). Altogether, our present results and these observations argue against a fundamental role of collybistin for clustering of gephyrin and other postsynaptic proteins. The dominant-negative effects produced by interference of collybistin-gephyrin interaction in cultured neurons (Harvey et

al., 2004) might therefore be secondary to a perturbation occurring downstream of collybistin, possibly mediated by deregulation of cdc42.

In contrast to our observations in the molecular layer of the cerebellum, the loss of gephyrin clustering leads to a drastic decrease of clusters of the synaptic $GABA_A$ receptor γ2-subunit in the hippocampus of adult collybistin-KO mice. The effect is seen not only in constitutive collybistin-KO mice, but also upon conditional deletion of the collybistin gene at early postnatal stages, using CaMKII-mediated Cre recombinase expression (Papadopoulos et al., 2008). These results obtained in parallel to the present study confirm the constitutive absence of gephyrin clustering upon genetic ablation of the collybistin gene.

It has been shown in hippocampal primary cultured neurons that gephyrin down-regulation affects the number of $GABA_A$ receptor clusters as well as the density of GABAergic synapses (Levi et al., 2004; Li et al., 2005; Fang et al., 2006; Yu et al., 2007) and that overexpression of mutant forms of gephyrin with increased propensity to form clusters leads to an increase of GABAergic postsynaptic clusters (Lardi-Studler et al., 2007). While suggesting that gephyrin itself might regulate GABAergic synaptogenesis, these observations need to be reconciled with the present results that GABAergic synaptogenesis proceeds unperturbed in the absence of gephyrin clustering in Purkinje cells. To this end, a better understanding of the interaction between gephyrin and NL2, as well as gephyrin and specific $GABA_A$ receptor subtypes, will be required.

The proper postsynaptic clustering of NL2 in the collybistin-KO mice underscores previous findings that NL2 forms clusters at presumptive GABAergic postsynaptic sites in Purkinje cells devoid of $GABA_A$ receptors (Patrizi et al., 2008). Therefore, it is conceivable that NL2 governs gephyrin and/or $GABA_A$ receptor postsynaptic accumulation, possibly by direct interaction or by favoring stabilization of presynaptic terminals via neurexin interactions, leading to release of a putative clustering signal. To test this hypothesis, it would be interesting to investigate formation of GABAergic synapses upon interference of NL2-neurexin interaction.

Altogether, we conclude here that in cerebellar Purkinje cells different steps of GABAergic synapse formation and maintenance until adulthood does not depend on collybistin-dependent gephyrin clustering. Whether absence of gephyrin in Purkinje cell synapses has

functional consequences on GABAergic function cannot be inferred from the present studies and should be investigated electrophysiologically.

2.6. Acknowledgments

We would like to thank Corinne Sidler for technical assistance and help with histology. This work was supported by the Swiss National Science Foundation (grant Nr. 3100A0-108260 to JMF).

3. Study III: Developmental maturation of synaptic and extra-synaptic $GABA_A$ receptors in mouse thalamic ventro-basal neurons

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Dianne R. Peden, Caroline M. Petitjean and Murray B. Herd contributed equally to this work

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The morphological part of the original publication was extracted and is presented here

3.1. Abstract

Thalamic ventrobasal (VB) relay neurons express multiple $GABA_A$ receptor subtypes mediating phasic and tonic inhibition. During postnatal development, marked changes in subunit expression occur, presumably reflecting changes in functional properties of neuronal networks. Thus, synaptic $GABA_A$ receptors in the immature brain incorporate the α2-subunit. After the second postnatal week they are replaced by α1-subunit containing receptors. In mice lacking the α1-subunit gene ($\alpha_1^{0/0}$), mIPSCs disappear during neuronal maturation, indicating that α2-$GABA_A$ receptors are not replaced. Here, we studied the morphological consequences of $GABA_A$ receptor loss in $\alpha_1^{0/0}$ mice during postnatal development. Using immunohistochemistry and transmission electron microscopy, we found that the loss of synaptic $GABA_A$ receptors led to a delayed disruption of gephyrin clusters and GABAergic postsynaptic densities. Despite these alterations, GABAergic presynaptic terminals were preserved and likely maintaining tonic inhibition. Intriguingly, in adult VB neurons of $\alpha_1^{0/0}$ mice, the preserved terminals were found to establish non-synaptic junctions, similar to those normally found between glutamatergic presynaptic terminals and dendrites. The enrichment of synaptic vesicles at these junctions suggests that they compensate for the loss of transmitter release sites at synapses.

3.2. Introduction

$GABA_A$ receptors mediate fast GABAergic neurotransmission, and are assembled from a large family of subunits (Barnard et al., 1998). $GABA_A$ receptors differing in subunit composition are distinguished by their function, pharmacology, subcellular localization (*e.g.* synaptic *versus* extrasynaptic) and spatio-temporal expression patterns (Fritschy and Brunig, 2003; Korpi and Sinkkonen, 2006). This heterogeneity is exemplified in the adult rodent thalamus, which contains at least three major $GABA_A$ receptor populations (Wisden et al., 1992; Fritschy and Mohler, 1995; Zhang et al., 1997). In relay nuclei, such as VB, or the lateral geniculate nucleus (LGN), synaptic ($\alpha1\beta2\gamma2$) and extrasynaptic ($\alpha4\beta2\delta$)-$GABA_A$ receptors coexist, whereas the reticular nucleus (nRT) and intralaminar nuclei mainly express synaptic $\alpha3\beta3\gamma2$ $GABA_A$ receptors. These receptor populations have defined functional and pharmacological properties, with $\alpha1$- and $\alpha3$-$GABA_A$ receptors mediating phasic inhibition, whereas $\alpha4\delta$-$GABA_A$ receptors mediate tonic inhibition (Farrant and Nusser, 2005; Jia et al., 2005; Chandra et al., 2006; Bright et al., 2007). At the network level, these distinct $GABA_A$ receptors contribute to the regulation of thalamocortical rhythmic activity associated with sleep, wakefulness and vigilance and are implicated in seizure disorders (Huntsman and Huguenard, 2000; Sohal et al., 2003).

The $GABA_A$ receptor subtypes of relay nuclei have a delayed expression during postnatal development. In rodents at birth, expression of $\alpha1$, $\alpha4$, $\beta2$, and δ subunits is low and relay neurons in the VB and LGN mainly express $\alpha2$ and $\beta3$ subunits (Laurie et al., 1992b; Fritschy et al., 1994). An apparent switch in expression occurs during brain maturation, with $\alpha1$- gradually replacing $\alpha2$-$GABA_A$ receptors, a re-organization proposed to influence the kinetics of IPSCs (Okada et al., 2000). In contrast, in the nRT, $\alpha3$-$GABA_A$ receptors are abundant at every postnatal age examined, although a transient expression of the $\alpha5$ subunit around P7 has been reported (Studer et al., 2006). The presence of multiple $GABA_A$ receptor subtypes within single neurons ($\alpha2$- in immature and $\alpha1$- and $\alpha4$-$GABA_A$ receptors in mature relay neurons) raises the question of how their assembly and subcellular targeting are regulated, and to what extent their function is determined by subunit composition. Thus, developing VB neurons represent an excellent model to address these fundamental questions.

Here, we investigated the plasticity of $GABA_A$ receptors in VB neurons during postnatal ontogeny using morphological approaches in wild type (WT) and α1 subunit null ($\alpha_1^{0/0}$) mice (Vicini et al., 2001). As described in Peden et al., (2008), the mIPSCs of $\alpha_1^{0/0}$ VB neurons disappeared during maturation, indicating that the α2 subunit was not replaced. Therefore we used immunohistochemistry for $GABA_A$ receptor subunits and pre- and post-synaptic markers of GABAergic transmission to determine how this loss of function affects GABAergic synapses in VB neurons of $\alpha_1^{0/0}$ mice as well as electron microscopy to study the effect at the ultrastructural level. In line with the electrophysiological results, we found a gradual loss of α2-subunit clusters which were not replaced by another synaptic subunit. Gephyrin remained clustered at non-functional synaptic sites for several weeks while presynaptic terminals were preserved up to adulthood. Intriguingly, the disruption of synaptic transmission caused by deletion of the α_1 subunit had no effect on extrasynaptic receptor expression which increased with development (P8 – 27) similarly for VB neurons derived from WT and $\alpha_1^{0/0}$ mice. In electron micrographs of adult $\alpha_1^{0/0}$ mice, we found a loss of postsynaptic densities at presynaptic terminals positive for GABA staining. Instead, such terminals were found to be connected to electron dense structures resembling filamentous contacts, a type of non-synaptic junction characteristic for presynaptic terminals onto dendrites of relay neurons in the thalamus (Lieberman and Spacek, 1997).

3.3. Materials and Methods

All experiments have been approved by the local authorities and were performed in accordance with European Community Council Directive 86/609/ EEC and with the institutional guidelines of the Universities of Zurich and Dundee.

Immunohistochemistry

All morphological experiments were performed on WT and $\alpha_1^{0/0}$ mice generated on a mixed C57BL/6J-129Sv/SvJ at the University of Pittsburgh (Vicini et al., 2001) and obtained from heterozygous ($\alpha_1^{+/0}$) breeding pairs, or from first generation WT and $\alpha_1^{0/0}$ breeding pairs derived from $\alpha_1^{+/0}$ mice. For the illustration of GABAergic neurons in the thalamus, sections of GAD67-EGFP knock-in mice (for characterization see Tamamaki et al., (2003) bred on a heterozygous background by crossing with C57BL/6J mice were used. Six different sets of experiments were performed using the following antibodies: Guinea pig antisera against $GABA_A$ receptor subunits α1, α2, α3 and γ2 (raised in house); rabbit antibodies against the α4 (PhosphoSolutions, Aurora, CO; cat. no. 844-GA4N) and δ subunit (Chemicon International, Temecula, CA; cat. no. AB9752), the vesicular GABA transporter (VGAT; Synaptic Systems, Göttingen, Germany, cat. no. 131003); mouse monoclonal antibody 7a against gephyrin (Synaptic Systems, cat. no. 147011); Rabbit antiserum against GABA (Sigma, St. Louis, MO); rabbit antibody against EGFP (Synaptic Systems). The characterization of antibodies was based on previous results (Kralic et al., 2006; Studer et al., 2006) and on their known regional and cellular expression pattern.

Immunoperoxidase staining

The distribution of $GABA_A$ receptor subunits in the thalamus was compared in WT and $\alpha_1^{0/0}$ mice during postnatal development (P10, P20, P30, P60, n=3 per age and genotype) in sections processed for immunoperoxidase staining. Mice were deeply anesthetized with "Nembutal" (50 mg/kg, i.p.) and transcardially perfused with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.15 M phosphate buffer, pH 7.4. The brains were extracted immediately after the perfusion and postfixed in the same solution (P10, 24-36 hr; P20, 12 hr; P30 and adult, 4-6 hr). Tissue was then processed for antigen

retrieval as described (Kralic et al., 2006), cryoprotected by with 30% sucrose in PBS, and cut parasagitally at 40 μm from frozen tissue with a sliding microtome. Sections were collected in PBS and stored in antifreeze solution (15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) prior to use.

Sections were incubated free-floating overnight at 4°C with primary antibodies against $GABA_A$ receptor subunits (α1, 1:20,000; α2, α4, δ, affinity purified, 1-2 μg/ml; α3, γ2,1:3,000) in Tris buffer containing 2% normal goat serum and 0.2% Triton X-100. Sections were then washed and incubated for 30 min at room temperature with biotinylated secondary antibodies (1:300; Jackson Immunoresearch, West Grove, PA), followed by incubation in avidin-biotin complex (1:100 in Tris buffer) for 30 min (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA), washed again and finally reacted with diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The color reaction was stopped after 5-15 min with "ice-cold" PBS. Sections were then mounted on gelatin-coated slides and air-dried. Finally, they were dehydrated with ethanol, cleared in xylol, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). Sections from wildtype and $\alpha_1^{0/0}$ mice were processed in parallel under identical conditions to minimize variability in staining intensity.

Immunofluorescence staining

Co-localization of $GABA_A$ receptor α1, α2 α4, and γ2 subunit with gephyrin at presumptive postsynaptic sites was analyzed in WT and $\alpha_1^{0/0}$ mice using double immunofluorescence staining in sections prepared from fresh-frozen tissue. Mice (P10-12, P15, P20, P30; n=3-5 per age and per genotype) were anesthetized with isoflurane, decapitated, and the brain extracted rapidly and frozen with powdered dry ice. Transverse sections were cut at 12 μm with a cryostat, mounted onto gelatin-coated glass slides, air dried at room temperature for 1 min, and stored at –20°C. They were then thawed at room temperature and fixed in methanol at –20°C for 2 min After 2 washes in PBS, the sections were incubated overnight at 4°C with a mixture of primary antibodies diluted in PBS containing 4% normal goat serum. Sections were washed extensively in PBS and incubated for 30 min at room temperature with the corresponding secondary antibodies conjugated to Cy3

(1:500, Jackson Immunoresearch) or Alexa488 (1:1000, Molecular Probes, Eugene, OR). Sections were washed again with PBS and coverslipped with mounting medium (DAKO, Carpinteria, CA).

For co-visualization of presynaptic terminals and gephyrin clusters in WT and $\alpha_1^{0/0}$ mice (P30, P60, n=3 per age and genotype), tissue was prepared as described (Schneider Gasser et al., 2006). Mice were decapitated as above, the brain rapidly removed and placed in oxygenated "ice-cold" artificial cerebral spinal fluid (aCSF). Slices were prepared with a vibratome and incubated for 20 min in aCSF at 34°C. They were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, extensively washed and incubated overnight in 30% sucrose in PBS for cryoprotection. Transverse sections (16 μm) were cut from frozen slices, mounted onto gelatin-coated slides, air dried at room temperature for 1 min, and stored at –20°C. They were then processed for double immunofluorescence staining with antibodies to VGAT (1:3,000) and gephyrin (1:1,000) as described above.

For quantification of GABAergic terminals in the VB of wildtype and $\alpha_1^{0/0}$ mice (P10, P30, P60, n=3 per age and genotype), immunofluorescence staining was performed with anti-VGAT antibodies in perfusion-fixed tissue that was not processed for antigen retrieval (see immunoperoxidase staining, above). After overnight incubation in primary antibodies and washing, free-floating sections were incubated with secondary antibodies conjugated to Cy3 in Tris buffer containing 2% normal goat serum for 30 min at room temperature, washed and coverslipped with DAKO mounting medium.

Preembedding immunoelectron microscopy

Visualization of gephyrin clusters at ultrastructural level in 1 month old animals was achieved with preembedding staining for gephyrin in tissue prepared for electron microscopy. P30 wildtype and $\alpha_1^{0/0}$ mice (n=3 per genotype) were perfused as above with a fixative containing PBS, 4% paraformaldehyde, 0.1% glutaraldehyde and 15% picric acid. The brains were removed and postfixed in the same fixative for 6 h and washed with phosphate buffer (PB). 70 μm coronal sections were cut on a vibratome and VBs were dissected and cryoprotected in PB containing 30% succrose over night at 4°C. To enhance antibody penetration, the sections were frozen and thawed rapidly 3 times using liquid

nitrogen. They were then collected and washed in TBS, and blocked for 1 h in TBS containing 10% NGS. The first antibody directed against gephyrin was applied for 72 h at 4°C in TBS containing 3%NGS and 0.05% sodium azide to prevent bacterial contamination. The sections were then incubated over night at 4°C in PBS containing 3% NGS and the secondary antibody conjugated to biotin (1:250, Jackson Immunoresearch). The immunoperoxidase reaction was performed as described above. The sections were then collected in cacodylate buffer (0.1 M $Na(CH_3)_2AsO_2*3H_2O$, pH 7.4, EMS, Hatfield, PA, USA), postfixed in 2.5% glutaraldehyde in cacodylate buffer for 2 h at 4°C. The immunoperoxidase reaction product was silver-intensified with an aqueous solution containing 3% hexamethylenetetramine (Merck 4343), 0.25% silver nitrate (Merck 1512) and 0.025% Borax (Merck 6310) for 10 min at 60°C and gold-toned with an 0.05% gold chloride aqueous solution (Merck 1582) for 2 min at room temperature. Sections were then incubated in 2.5% sodium thiosulfate for 2 min at room temperature and washed in cacodylate buffer. Osmification was performed in a 0.5% osmium tetroxide solution on ice. For contrast enhancement, a 1% aqueous solution of uranyl acetate was applied for 30 min at room temperature. Finally, the sections were dehydrated with increasing acetone concentration and flat-embedded in Epon812 (Giustetto et al., 1998). The Epon812 polymerization was achieved by incubation at 60°C for 48 h.

50 nm ultrathin sections were cut on a ultramicrotome (Leica EM UC6, Leica Microsystems) and mounted on pioloform (Plano GmBH, Wetzlar, DE) coated nickel grids. Before imaging, ultrathin sections were further contrasted in a 2% lead-citrate solution for 10 min at room temperature.

Postembedding immunoelectron microscopy

For ultrastructural visualization of GABAergic terminals in 2 month-old mice, tissue was prepared for electron microscopy and postembedding staining with an antibody directed against GABA was used. P60 WT and $\alpha_1^{0/0}$ mice (n=2 per genotype) were perfused as above with a fixative containing PBS with 4% PFA, 15% picric acid and 1% glutaraldehyde, brains were removed and postfixed for 6 h. 70 μm coronal sections were cut at a vibratome and VBs were dissected and collected in cacodylate buffer. Osmification, dehydration and embedding in Epon812 was performed as described above.

The postembedding staining was performed on 70 nm ultrathin section mounted onto pioloform-coated nickel grids. Etching of the Epon812 was achieved by exposing the sections to an aqueous solution containing 1% sodium meta periodate (Merck 0050) for 1 min, collected in TBS-T (TBS containing 0.1% Triton X-100) and further exposure to a 0.2% sodium borohydride (Fluka) solution in TBS-T and 50 mM glycine. The sections then were blocked for 10 min in 2% human serum albumin (HSA) in TBS-T and incubated in the same solution containing the first antibody (GABA, 1:1000) over night. Sections were washed in TBS-T, blocked for 10 min in TBS-T/2%HSA and exposed to the secondary antibody conjugated to 10 nm gold particles (1:10, BBInternational, Cardiff, UK) in TBS-T/2%HSA containing 0.5mg/ml polyethyleneglycol MW 15-20 kD (Sigma) for 1 h. After washing in TBS-T and H_2O, the contrasting was performed with 1% Uranyl acetate and 2% lead citrate as described above.

Image analysis

Sections processed for immunoperoxidase staining were visualized with brightfield microscopy (Axioplan; Zeiss) using the mosaic software (Explora Nova, La Rochelle, France) for image acquisition. For each antibody, similar acquisition parameters were selected for WT and $\alpha_1^{0/0}$ animals. Images were cropped to the desired dimensions in Adobe Photoshop CS. Minimal adjustments of contrast and brightness were made to entire images, if needed.

Double-immunofluorescence staining was visualized by confocal microscopy (Zeiss LSM-510 Meta; Jena, Germany) using a 100x Plan-Apochromat objective (N.A. 1.4). The pinhole was set to 1 Airy unit for each channel and separate color channels were acquired sequentially. The acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. For display, images were processed with the image-analysis program Imaris (Bitplane; Zurich, Switzerland). Images from both channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. A low-pass "edge preserving" filter was used for images displaying α_1, α_2 or VGAT staining.

Postsynaptic clustering of gephyrin and gephyrin/α subunit co-localization was quantified from single confocal sections (512 x 512 pixels) at a magnification of 90 nm/pixel in 8 bit grayscale images, using a threshold segmentation algorithm (minimal intensity, 90-130;

size 0.08 – 0.8 μm^2). From the gephyrin-α subunit co-localized images all structures > 0.057 μm^2 were counted, representing a minimum of 70% overlay of both channels to be considered as co-localized (ImageJ imaging software, NIH, Bethesda, MD). The data was quantified in 6 sections per animal (n=3 per genotype and age, Kruskal-Wallis followed by Dunn's Multiple Comparison Test, Prism; GraphPad, San Diego, CA).

For quantification of the density of GABAergic presynaptic terminals, volumes were reconstructed from stacks of 13 confocal images (512 x 512 pixels) spaced by 0.25 μm at a magnification of 90 nm/pixel. All files were blinded and individual terminals were identified as isolated objects with 1 μm^3 minimal apparent volume and counted automatically (Imaris; Bitplane). Size distribution of VGAT positive terminals was analyzed in single confocal sections (ImageJ imaging software, NIH, Bethesda, MD). These data were quantified in 6 sections per animal (n=3 per genotype and age, Kolmogorov-Smirnov test).

For electron microscopy, ultrathin sections were examined in a Philips CM100 electron microscope equipped with a side-mounted CCD camera (Gatan Bioscan, Pleasanton, CA, USA). Each site of interest was sampled in at least 3 consecutive sections. Images were cropped and contrast was adjusted when necessary in ImageJ.

3.4. Results

Unaltered developmental maturation of the α2 subunit in VB of $\alpha_1^{0/0}$ mice

The developmental changes in the properties of synaptic $GABA_A$ receptors in VB neurons were mirrored in WT mice by the delayed expression of the α1 subunit, which was almost undetectable immunohistochemically at P10, but exhibited a prominent staining at P20 (Figure 1A, G). In parallel, immunoreactivity for the α2 subunit, which was very strong at birth (not shown), decreased gradually to disappear between P10 and P20 (Figure 1B, H). These changes are in striking contrast to the α3 subunit-immunoreactivity, which selectively labeled the nRT (Studer et al., 2006) and remained rather constant throughout postnatal development (Figure 1C and I). In $\alpha_1^{0/0}$ mice, the developmental maturation of the α2 and α3 subunit was the same as in WT (Figure 1, middle and right column), and the absence of the α1 subunit was not compensated for by these subunits. These observations are in line with the complete loss of synaptic $GABA_A$ receptor-mediated currents in VB neurons of $\alpha_1^{0/0}$ mice (Peden et al., 2008).

Synaptic $GABA_A$ receptors are associated with gephyrin

Gephyrin is a marker of GABAergic and glycinergic postsynaptic sites in the CNS, forming clusters that are selectively co-localized with $GABA_A$ receptor subunits, as seen by immunofluorescence staining (Sassoe-Pognetto et al., 2000; Kralic et al., 2006). A systematic analysis of gephyrin clusters and their co-localization with the α1 and α2 subunit was therefore conducted between P10 and P30 in the VB of both WT and $\alpha_1^{0/0}$ mice. At P10, a high density of gephyrin clusters was evident, with almost 80% of them being co-localized with the α2 subunit in both genotypes and less than 10% with the α1 subunit in WT (Figure 2A, B, I). The density of gephyrin clusters was the same in sections from mutant and WT (Figure 2J). The incidence of co-localization between gephyrin clusters and the α2 subunit decreased gradually by about 10% per day to become negligible at P20 (Figure 2A-F, I, $p<0.05$ in both genotypes, Kruskal-Wallis test), reflecting the gradual disappearance of the α2 subunit immunoreactivity and the loss of synaptic GABAergic currents recorded in neurons from mutant mice (Peden et al., 2008). In WT

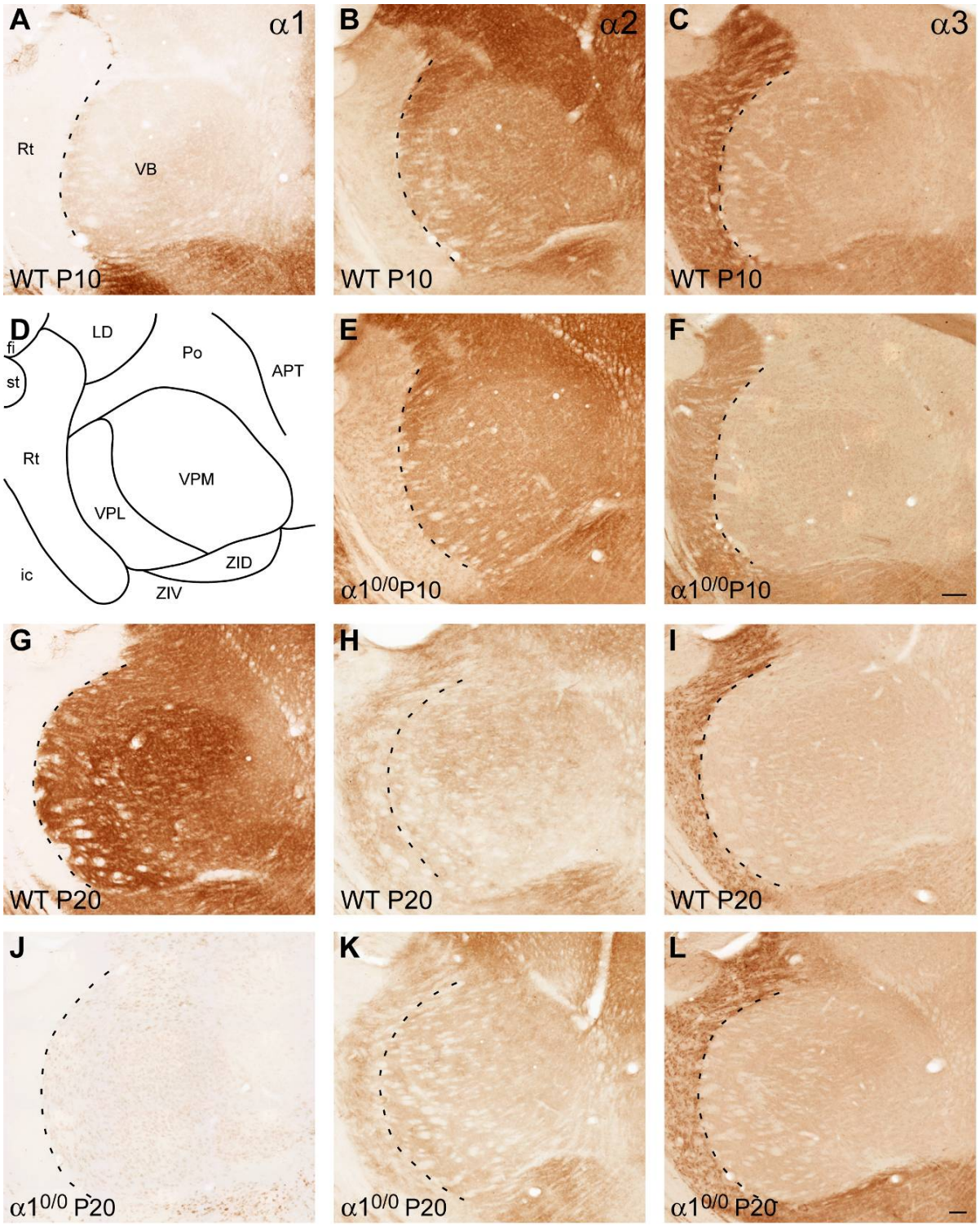


Figure 1

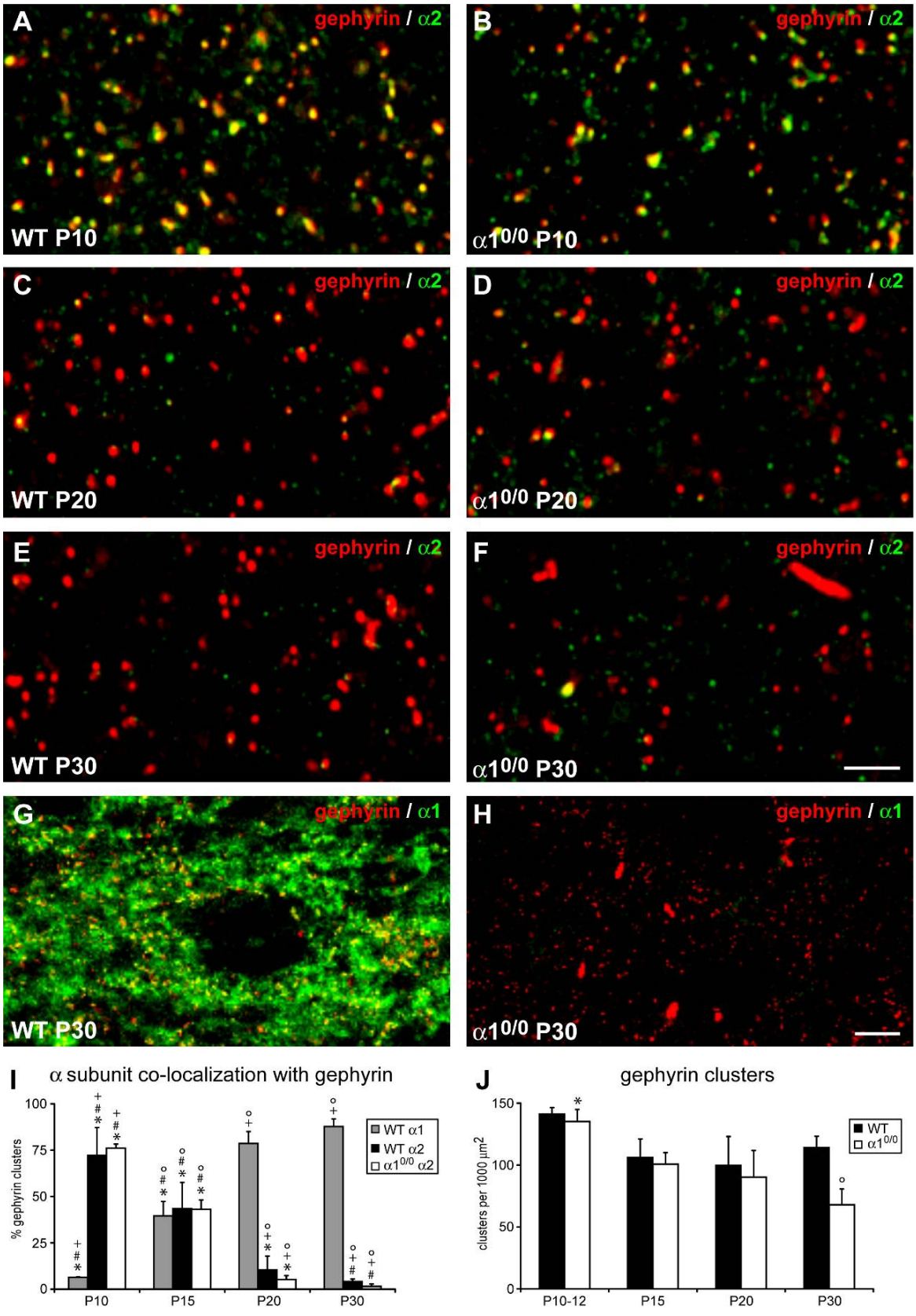
Comparative distribution of α1 (A, G, J), α2 (B, E, H, K), and α3 (C, F, I, L) subunit immunoreactivity in the thalamus at P10 (A-C, E, F) and P20 (G-L) in WT (A-C, G-I) and $\alpha_1^{0/0}$ (E, F, J-L) mice.

neurons, co-localization with the α1 subunit was around 40% at P12-P15 and abruptly increased to almost 100% at P20 (Figure 2I, $p<0.05$, Kruskal-Wallis test). We could not determine whether α1-GABA$_A$ receptors replace α2-GABA$_A$ receptors at the same synaptic sites, or whether the switch in subunit expression reflects the formation of novel synapses. However, the conservation of gephyrin clusters in VB neurons from $\alpha_1^{0/0}$ mice, most likely devoid of associated GABA$_A$ receptors, favors the first alternative.

Delayed alteration of gephyrin clustering in VB neurons of $\alpha_1^{0/0}$ mice

Between P10 and P30, there was a significant reduction in gephyrin cluster density with increasing age in WT and $\alpha_1^{0/0}$ animals ($p<0.01$ in both genotypes, Kruskal-Wallis), reflecting the growth of the VB (Figure 2J). At P30, the density of gephyrin clusters in WT mice was 20% lower than at P10 (n.s.), whereas in $\alpha_1^{0/0}$ mice the reduction reached 50% ($p<0.05$), reflecting the absence of association with the α1 subunit. Instead, large, presumably intracellular, aggregates became apparent, as described previously in sections from adult mice (Kralic et al., 2006). This trend continued at later stages, with gephyrin clusters being replaced by large intracellular aggregates until about P60. At this age, no gephyrin clusters remained in the VB of mutant mice. In view of the complete absence of mIPSCs in VB neurons from mutant mice at P23 and later (Peden et al., 2008), we interpret this trend as evidence for impaired gephyrin postsynaptic clustering in the absence of associated GABA$_A$ receptors.

Parasagittal sections were processed for immunoperoxidase staining. A schematic drawing of the regions depicted is given in panel **D**. The α1 subunit is conspicuously absent in VB (VPL + VPM) of P10 WT mice, but increases rapidly thereafter. In contrast, the α2 subunit staining is moderate at P10 and decreases to background levels by P20 in both WT and $\alpha_1^{0/0}$ mice. Finally, the α3 subunit immunoreactivity, which is intense in Rt in both genotypes, is not detectable in VB at either age. Abbreviations: (APT) anterior pretectal nucleus, (fi) fimbria of the hippocampus, (ic) internal capsule, (LD) laterodorsal thalamic nucleus, (Po) posterior thalamic nuclear group, (Rt) reticular thalamic nucleus, (st) stria terminalis, (VPL) ventral posterolateral thalamic nucleus, (VPM) ventral posteromedial thalamic nucleus, (ZID) zona incerta, dorsal part, (ZIV) zona incerta, ventral part. Scale bar = 100 μm (scale bar in F applies to A-F, scale bar in L applies to G-L).


A
gephyrin / α2
WT P10
B
gephyrin / α2
α1 0/0 P10
C
gephyrin / α2
WT P20
D
gephyrin / α2
α1 0/0 P20
E
gephyrin / α2
WT P30
F
gephyrin / α2
α1 0/0 P30
G
gephyrin / α1
WT P30
H
gephyrin / α1
α1 0/0 P30
I
α subunit co-localization with gephyrin
% gephyrin clusters
100
75
50
25
0
P10
P15
P20
P30
WT α1
WT α2
α1 0/0 α2
J
gephyrin clusters
clusters per 1000 µm2
150
100
50
0
P10-12
P15
P20
P30
WT
α1 0/0

At the subcellular level, the α2 subunit-immunoreactivity in sections from P10 and P20 mice was punctate, forming clusters extensively co-localized with gephyrin (Figure 2A-D). In contrast, the α1 subunit staining was diffuse in the neuropil, outlining the cell body of individual VB neurons (Figure 2G). Although most gephyrin clusters were double-labeled for the α1 subunit, the majority of α1 subunit staining was not associated with gephyrin. While such a pattern is suggestive of extrasynaptic $GABA_A$ receptor labeling, it is also possible that most of the α1 subunit-immunoreactivity reflects intracellular pools of subunit protein (or assembled receptors).

Normal maturation and subcellular localization of subunits forming extrasynaptic $GABA_A$ receptors

Between P10 and P20, there is a increase of tonic inhibition in the thalamus, as has been measured by electrophysiology (Peden et al., 2008). Here this increase was mirrored by a developmentally-regulated expression of the α4 subunit in the VB, as detected by immunoperoxidase staining at these stages (Figure 3A-D). No difference in distribution or staining intensity was evident between WT and $\alpha_1^{0/0}$ mice (compare Figure 3A-B at P10 and Figure 3C-D at P20). Similar findings were obtained for the δ subunit (not shown), which has a very similar distribution to that of the α4 subunit in VB. On the subcellular

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Figure 2

Differential alterations in postsynaptic clustering of the α2 (green; A-F) and α1 subunit (green; G-H) and gephyrin (red, A-H) during development of WT (left column) and $\alpha_1^{0/0}$ (right column) mice, visualized by immunofluorescence staining and confocal laser scanning microscopy.

At P10 (**A** and **B**) most gephyrin clusters are colocalized with the α_2 subunit in both genotypes. This fraction gradually decreases between P10 to P30 (**A-F**, quantified in **I**), due to the loss of α2 subunit staining, which is replaced by the α1 subunit in WT (**G**, quantified in **I**), but not in mutants (**H**). As a result, gephyrin clusters in $\alpha_1^{0/0}$ mice remain isolated, and at P30, gephyrin forms large aggregates (**F, H**). **I**: Quantification of colocalization patterns between gephyrin and the α1 or α2 subunit in WT and $\alpha_1^{0/0}$ mice. Pair-wise significant differences between age groups are indicated by symbols (* $p<0.05$ compared to P30; # $p<0.05$ compared to P20; + $p<0.05$ compared to P15; ° $p<0.05$ compared to P10). **J**: Quantification of gephyrin cluster density in WT and $\alpha_1^{0/0}$ mice. No change is evident in WT, whereas a 30% decrease occurs in mutants between P10 and P30; the same symbols are used as in panel I. Scale bar in F = 3μm (applies to A-F), in H = 10μm (applies to G, H).

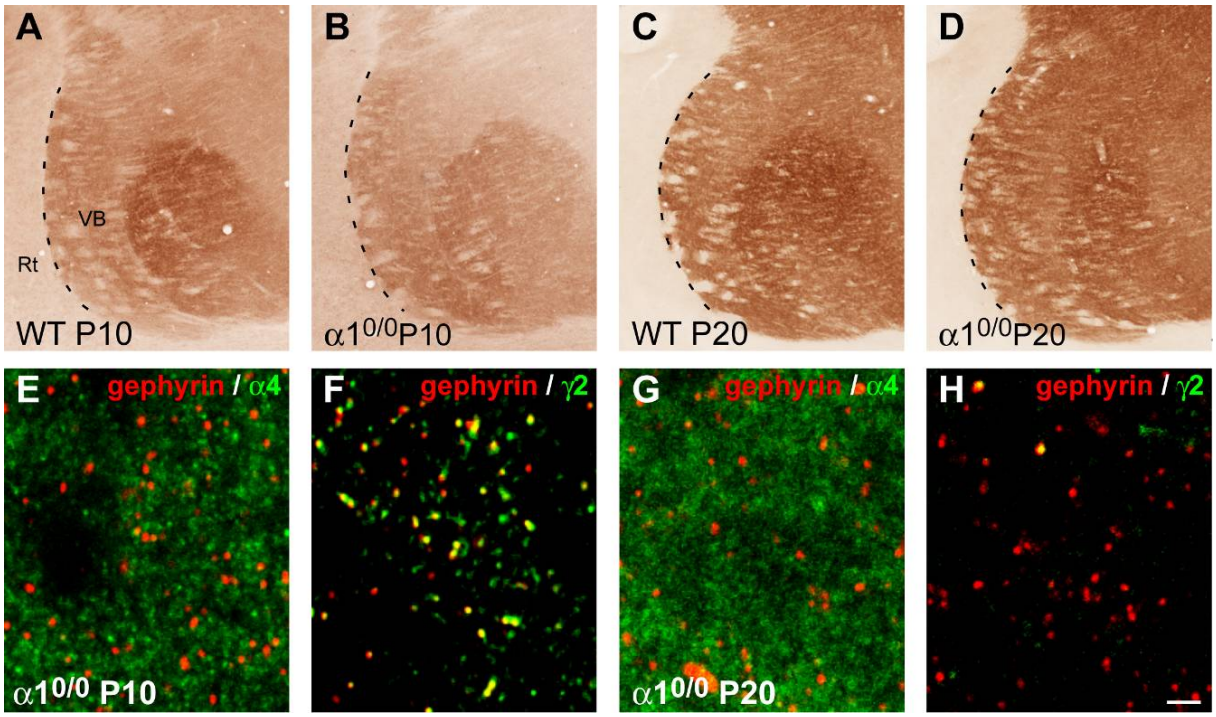

Figure 3

Comparative distribution of the α4 subunit immunoreactivity in VB of WT and $\alpha_1^{0/0}$ mice at P10 (A-B) and P20 (C-D), as illustrated by immunoperoxidase staining.
A, **B**, A moderate staining, selectively present in VB but not in the nRT, is already evident at P10, with a similar distribution in both genotypes. **C**, **D**, The staining intensity increases until P20, without revealing differences between WT and $\alpha_1^{0/0}$ mice at this stage. **E-H**, Double immunofluorescence for gephyrin (red) and the α4 (green; **E**, **G**) or γ2 (red; **F**, **H**) subunit at P10 and P20 in $\alpha_1^{0/0}$ mice; the extrasynaptic localization of the α4 subunit is inferred from its lack of colocalization with gephyrin, despite the absence of α1 subunit at either stage. Note that the γ2 subunit staining intensity decreases dramatically between P10 and P20, reflecting the loss of mIPSCs recorded electrophysiologically. Scale bars: D (applies for A-D), 100 μm; H (applies for E-H), 2 μm.

level, no co-localization between the α4 subunit and gephyrin was evident in sections from WT (not shown) and mutant mice (Figure 3E), whereas in the same tissue, γ2 subunit immunofluorescence formed bright clusters co-localized with gephyrin (Figure 3F). At P20, in line with the loss of postsynaptic $GABA_A$ receptors, the γ2 subunit was almost undetectable, whereas the α4 subunit immunoreactivity was diffuse in the neuropil (Figure 3G and H), as reported previously in adult mice (Kralic et al., 2006). These results confirm the independent regulation of α subunit variants and support functional data that α4-$GABA_A$ receptors do not contribute to synaptic transmission in VB neurons.

Long-term preservation of GABAergic terminals in VB of $\alpha_1^{0/0}$ mice

The disappearance of phasic $GABA_A$ receptor-mediated transmission and the maintenance of tonic inhibition raised the question to which extent GABAergic terminals are affected in the VB of $\alpha_1^{0/0}$ mice. Immunofluorescence staining for VGAT revealed no difference between genotypes at every age examined. At high-magnification, double labeling for VGAT and gephyrin showed that almost all gephyrin clusters were apposed to GABAergic terminals, which frequently appeared to form multiple postsynaptic sites (Figure 4A). In

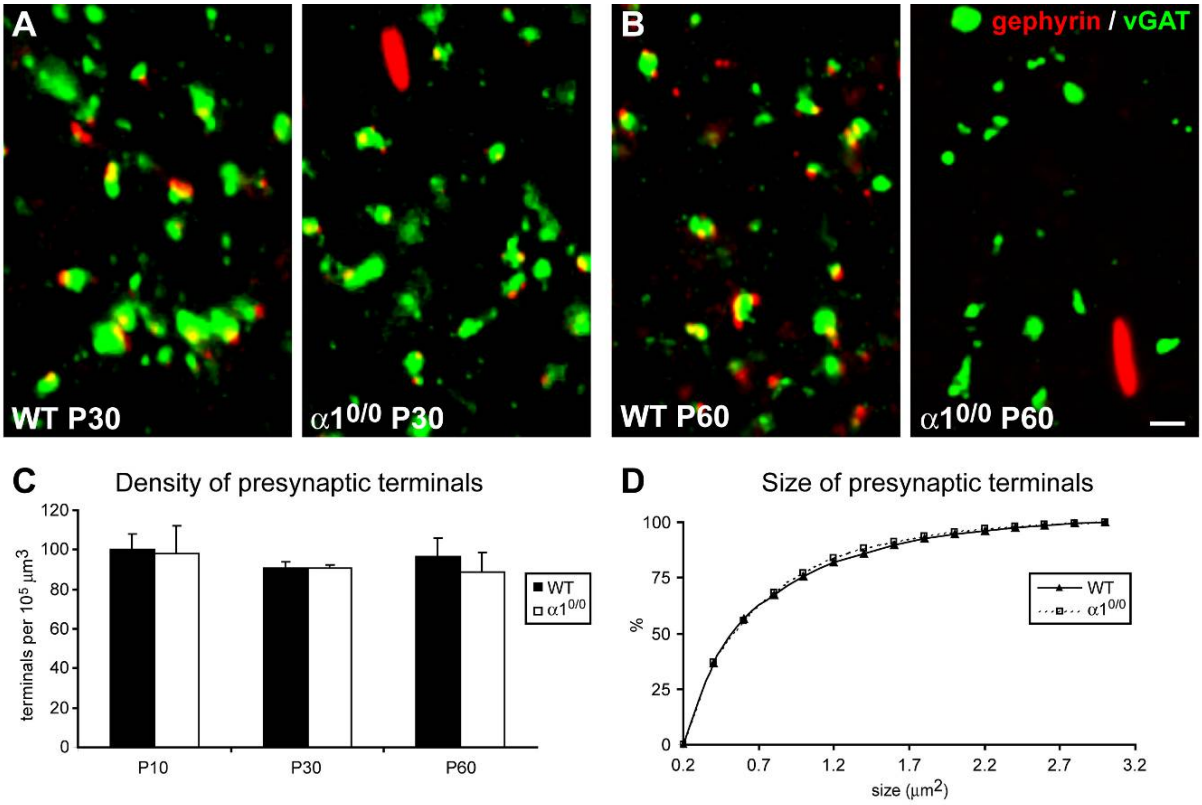

Figure 4

GABAergic terminals remain unaffected in VB of $\alpha_1^{0/0}$ mice during development, as seen by double immunofluorescence staining (A, B) of vGAT (green) and gephyrin (red).
A: At P30, the close apposition of both markers confirms the postsynaptic localization of gephyrin clusters in both genotypes, whereas large aggregates are not associated with gephyrin. **B**: At P60, most gephyrin clusters have disappeared and are replaced by aggregates. C: The density of vGAT positive terminals formed at P10 remains constant until adulthood and does not differ between WT and $\alpha_1^{0/0}$ mice. D: Likewise, their size, as determined by cumulative distribution analysis, is comparable in adult and WT $\alpha_1^{0/0}$ mice, despite the loss of postsynaptic proteins occurring in the mutants. Scale bar in B = 2 µm (applies to A and B).

sections from $\alpha_1^{0/0}$ mice, gephyrin intracellular aggregates were not apposed to VGAT-positive terminals, whereas the clusters remaining in the absence of α1 subunit were still apparently postsynaptic, as shown for a section of a P30 mouse (Figure 4B). A quantitative analysis confirmed these observations, showing no difference in the size of GABAergic terminals, as determined by cumulative distribution frequency analysis, or in their density, counted in three-dimensional volumes reconstructed from stacks of confocal images (Figure 4C and D). The preservation of GABAergic terminals was also seen with immunofluorescence for GAT1 (not shown), and may account for the maintenance of tonic inhibition in the VB of mutant mice.

Appearance of vesicle associated junctions at preserved GABAergic terminals

The preservation of GABAergic presynaptic terminals in spite of the loss of $GABA_A$ receptor and gephyrin clustering raised to the question how these changes affected the morphology of these terminals and whether they would form heterologous synapses with asymmetric postsynaptic densities, as observed in GABAergic terminals formed on Purkinje cells of $\alpha_1^{0/0}$ mice (Fritschy et al., 2006). We therefore performed immunogold labeling for GABA in 70 nm ultrathin sections of P60 WT and $\alpha_1^{0/0}$ VBs and analyzed at least three consecutive sections using transmission electron microscopy. In 2 WT animals, nearly all

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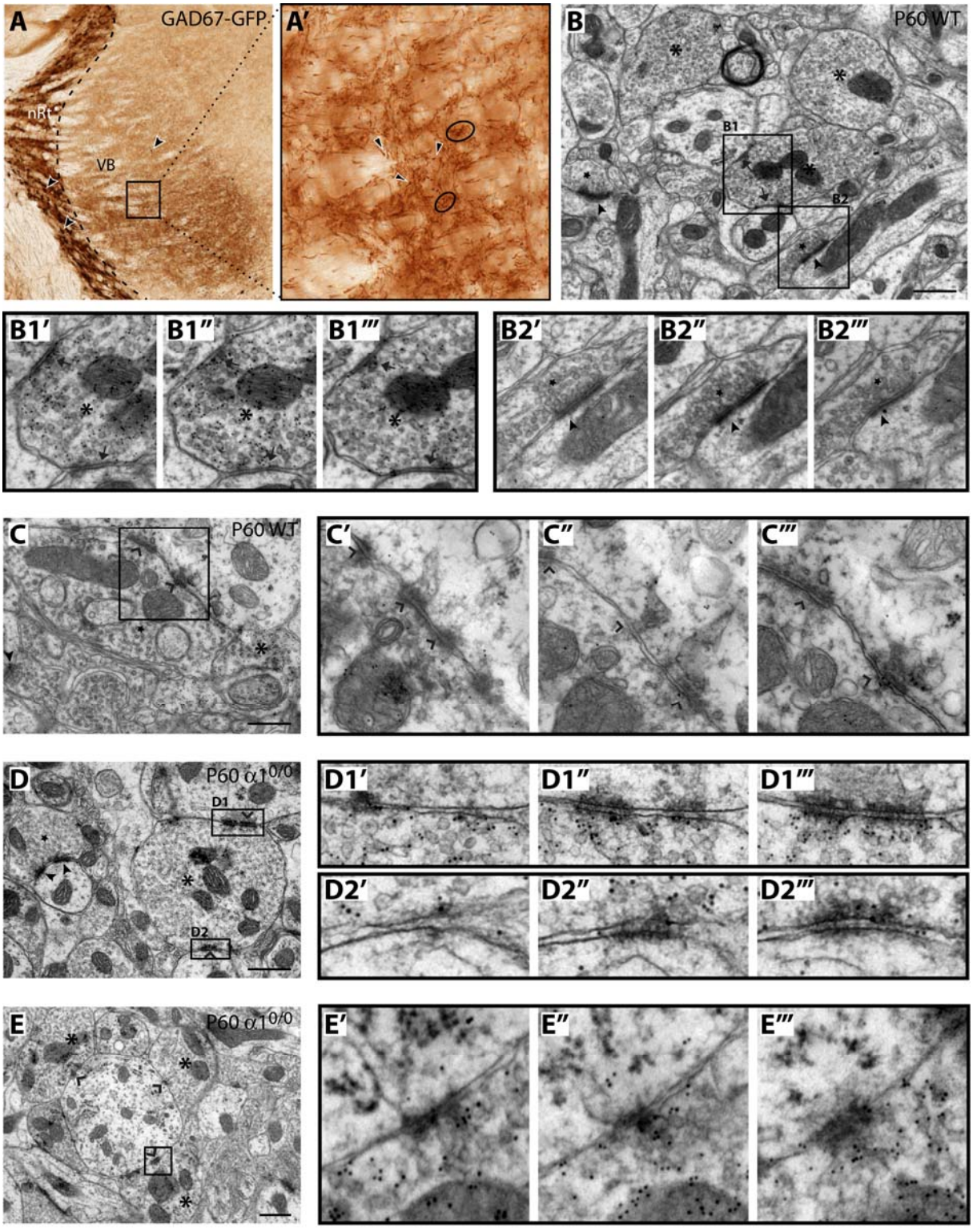
Figure 5

Formation of electron dense junctions with synaptic vesicle enrichment in GABA-positive terminals of P60 $\alpha_1^{0/0}$ mice, revealed by postembedding staining for GABA on ultrathin sections prepared for transmission electron microscopy.
A, A': Illustration of GABAergic innervation from the nRt into the VB in a GAD67-GFP mouse section in a DAB-staining for GFP. **A**: GABAergic cells (rounded arrowheads) located in the nRt project into the VB. Note the very few GABAergic interneurons in the VB illustrating that almost all GABAergic input in the VB is conducted from nRt axons. **A'**: At higher resolution the grouped arrangement of GABAergic terminals (arrowheads) in synaptic glomeruli (circles) are apparent. **B**: Example of grouped GABA-positive terminals (asterisk) in a synaptic glomerulus in P60 WT VB. Symmetric, presumably GABAergic synapses (arrows) are enlarged in three consecutive sections in panel **B1'-B1'''**. Asymmetric, presumably glutamatergic terminals are formed with GABA-negative terminals (star) and enlarged in three consecutive sections in panel **B2'-B2'''**. **C**: Illustration of filamentous contacts (corner) in P60 WT VB sections regularly formed by GABA-negative terminals onto dendrites, enlarged in consecutive sections in **C'-C'''**. Note that the junctions are devoid of



presynaptic vesicle enrichment. **D, E**: In P60 $\alpha_1^{0/0}$ VB sections, GABA-positive terminals regularly form electron dense structures (corners) with dendrites, resembling the contacts in **C**. **D1'-D1''', D2'-D2''', E'-E'''**: Enlarged panels of consecutive section of the contacts in **D** and **E**. Scalebar in B-E = 500 nm.

(in 18 out of 23) observed terminals positive for GABA labeling incorporated at least one GABAergic synapse. These were recognized by a continuous synaptic cleft, a synaptic vesicle enrichment at the presynaptic side and a pre- and postsynaptic electron dense band with similar densities, forming a symmetric synapse (Figure 5 B1). GABAergic terminals were most prominently found grouped in areas with a high concentration of synaptic terminals and synapses. Such synaptic glomeruli are a well described characteristic of thalamic relay nuclei and usually consist of a large lemniscal axon terminal (glutamatergic) forming synapses with invaginated dendritic excrescences of the target cells. Synaptic glomeruli additionally contain glutamatergic terminals originating from cortical afferents as well as GABAergic terminals (Spacek and Lieberman, 1974). Presynaptic terminals with only background GABA immunoreactivity were considered as GABA-negative and formed synapses with asymmetric postsynaptic densities, representing glutamatergic synapses (Figure 5 B2). In WT synaptic glomeruli, electron dense structures resembling characteristic thalamic junctions called "filamentous contacts" (Lieberman and Spacek, 1997) were regularly observed between GABA-negative (presumably glutamatergic) terminals and dendrites (Figure 5 B3). In the literature such junctions are described to also exist in GABAergic terminals, though considerably less frequent (Lieberman and Spacek, 1997). We never observed them here, which might be due to low number of terminals observed.

In $\alpha_1^{0/0}$ mice of the same age (P60), in none of the 40 GABA-positive terminals analyzed, a classical symmetric synapse was found. Instead, in 31 terminals we observed a prominent electron dense structure linking the terminal to a dendrite, distinctly resembling the non-synaptic junctions between glutamatergic terminals and dendrites described above (Figure 5D – E). Intriguingly, such junctions in the $\alpha_1^{0/0}$ mice contained synaptic vesicles at the presynaptic side, whereas classical junctions are not associated with vesicles. This

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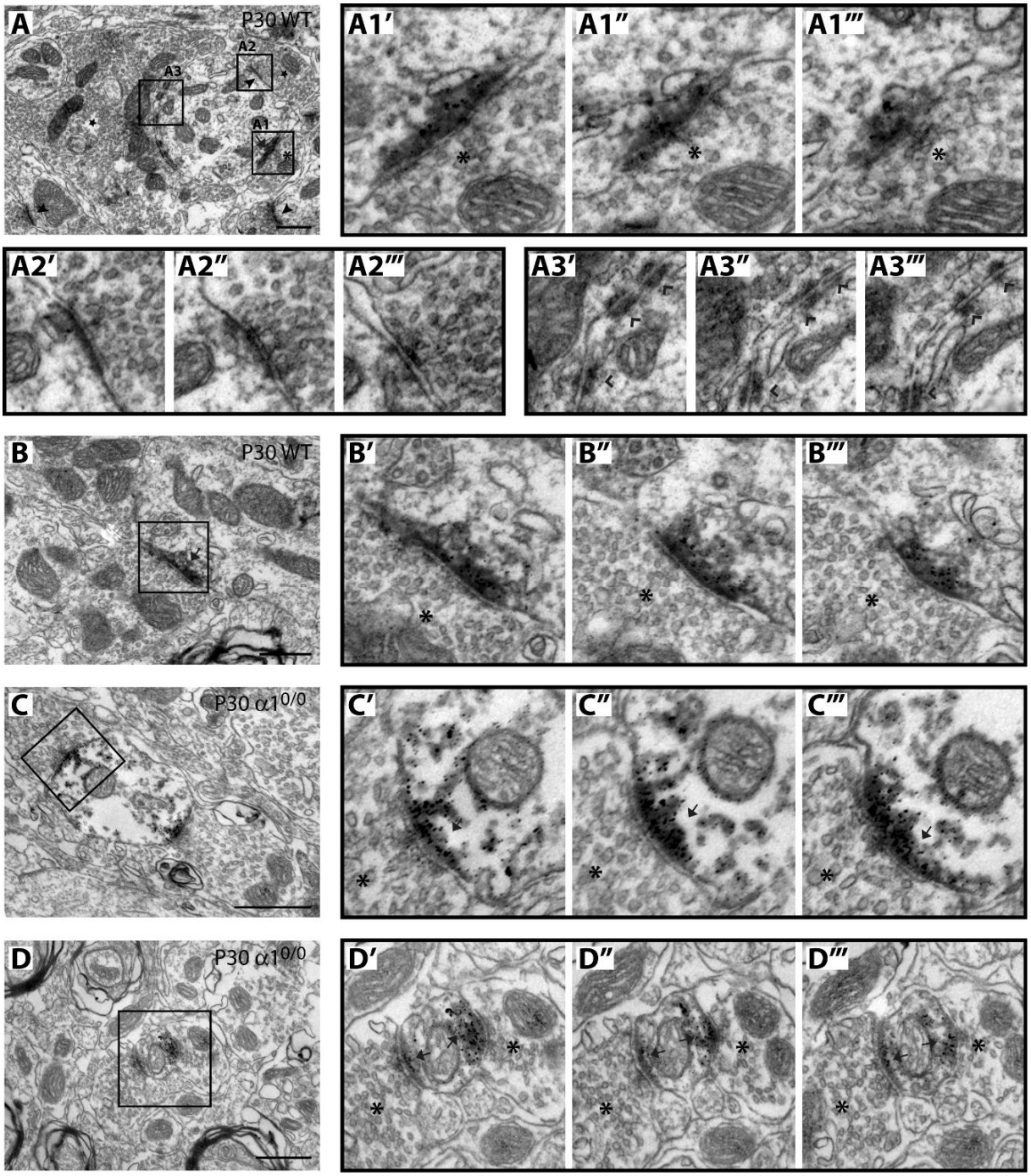
Figure 6

Gephyrin-positive postsynaptic clusters in P30 $\alpha_1^{0/0}$ mice are most abundant in small dendritic structures, as seen electron micrographs of preembedding staining for gephyrin.
A: Dendritic structure in a synaptic glomerulus of a P30 WT VB section, forming a symmetric GABAergic synapse, as indicated by positive gephyrin staining (arrow; the presumptive GABAergic terminal is marked with an asterisk), an asymmetric glutamatergic synapse (arrowhead; the



glutamatergic terminal is marked with a star), and a filamentous contact (corner) with a large presumably glutamatergic terminal. The GABAergic terminal (A1), the glutamatergic terminal (A2) and the contact (A3) are enlarged in three consecutive sections (**A1'-A1‴, A2'-A2‴, A3'-A3‴**). **B**: example of a typical GABAergic synapse positive labeled for gephyrin (arrow). Enlarged panels of consecutive sections in **B'-B‴**. **C-D**: In P30 $\alpha_1^{0/0}$ VB sections, gephyrin-positive staining (arrow) is mainly found at small dendritic structures, apposed to presumptive GABAergic terminals. **C'-C‴, D'-D‴**: Enlarged panels of consecutive section of the contacts in **C** and **D**. Scalebar = 500 nm.

suggests that these sites potentially contain neurotransmitter release sites, which might be necessary for the maintenance of tonic inhibition.

Remaining postsynaptic gephyrin staining apparent in 1 month old animals

To analyze if an intermediate morphological state of terminals can be observed in $\alpha_1^{0/0}$ mice, with GABAergic terminals being either associated to gephyrin-positive clusters or forming non-synaptic junctions, we examined the ultrastructural distribution of gephyrin in P30 WT and $\alpha_1^{0/0}$ mice. A preembedding staining for gephyrin was performed and analyzed at the transmission electron microscope. In P30 WT mice, we regularly observed gephyrin-positive postsynaptic densities apposed to vesicle-enriched terminals, representing GABAergic synapses (Figure 6 A1, B). As expected, they were predominantly localized in synaptic glomeruli. Also here, non-synaptic junctions between large, presumably glutamatergic, terminals and dendritic structures were regularly observed (Figure 6 A3). In P30 $\alpha_1^{0/0}$ animals, gephyrin-positive staining was sparsely found at postsynaptic sites, representing the decreasing fraction of GABAergic postsynaptic sites. Gephyrin-positive postsynaptic sites were mainly found at small dendritic structures and none of these terminals also formed non-synaptic junctions. We therefore speculate that the observed electron dense structures seen at P60 (Figure 5 D, E) are gephyrin negative.

3.5. Discussion

Two principal findings can be derived from this study. (1) The expression profile of each $GABA_A$ receptor subtype is independent of other subunits present in VB neurons, and no functional substitution occurs between subunits contributing to synaptic and extrasynaptic receptors. And (2) the maintenance of a gephyrin postsynaptic scaffold depends on the presence of synaptic $GABA_A$ receptors; however, presynaptic terminals are retained in the absence of functional synaptic transmission and are attached to dendrites with specialized junctions.

Development of synaptic $GABA_A$ receptors

GABAergic synapses in P8-9 VB relay neurons initially contain α2-$GABA_A$ receptors, clustered at presumptive postsynaptic sites with gephyrin. Inhibitory synapses of mature VB neurons express α1-$GABA_A$ receptors (Kralic et al., 2006) and the appearance of this subunit has been implicated in developmental changes to synaptic transmission (Okada et al., 2000; Goldstein et al., 2002; Ortinski et al., 2004; Bosman et al., 2005). However, P10 VB neurons exhibited little α1 subunit staining, with the majority of gephyrin clusters incorporating the α2 subunit.

During the next 10 days of development in VB neurons, the gephyrin/α2 subunit clusters undergo considerable reorganization so that by P20, nearly all such assemblies now contain the α1 subunit. Whether the subunit switch occurs within existing synapses, or whether synapses containing α2-$GABA_A$ receptors are replaced by novel ones incorporating the α1 subunit is not known. The maintenance of "orphan" gephyrin clusters for several weeks in the VB neurons of $\alpha_1^{0/0}$ mice favor the former scenario. The developmental loss of gephyrin-α2 subunit clusters occurs irrespective of the α1 subunit. In agreement, with subsequent development the proportion of $\alpha_1^{0/0}$ neurons in the VB exhibiting mIPSCs decrease, until P23-27 all neurons are "silent" (Peden et al., 2008). Evidently, these neurons (post P15) cannot stray from the developmental program and default to re-synthesize the α2 subunit. Although increased staining of the α4 subunit is evident in the VB neurons of $\alpha_1^{0/0}$ mice, this subunit does not cluster with gephyrin and

the absence of mIPSCs illustrates that this extrasynaptic subunit cannot deputize for the synaptic α1 subunit (Chandra et al., 2006; Kralic et al., 2006).

Mechanism of gephyrin clustering

Surprisingly, the gephyrin clusters of VB $\alpha_1^{0/0}$ mice remained for an extended time at presumptive postsynaptic sites devoid of functional $GABA_A$ receptors. In juvenile mice conditional knockout of the γ2 subunit in cerebral cortex and hippocampus caused gephyrin clusters to disappear within a few days (Schweizer et al., 2003). Although the γ2 subunit was not targeted in $\alpha_1^{0/0}$ mice, it also looses its postsynaptic localization, suggesting cell-specific differences in the stability of gephyrin clusters. Whether these differences reflect the molecular heterogeneity of gephyrin, or other components of GABAergic synapses is not established. In this regard, collybistin-null mice exhibit striking regional differences in the loss of postsynaptic gephyrin-$GABA_A$ receptor clusters in the absence of this GDP/GTP-exchange factor known to interact with gephyrin (Papadopoulos et al., 2007).

Development of GABAergic terminals

Although the mean absolute number of GABAergic synapses per VB neuron could not be quantified morphologically (using VGAT staining), an increase in synaptic coverage per relay neuron is likely, given the increased frequency of mIPSCs during development (Peden et al., 2008) and the similar density of gephyrin clusters seen in WT mice, despite the considerable growth of the neuropil in VB between birth and P30. Analysis of GABAergic terminals with VGAT revealed no detectable morphological alteration in VB neurons of $\alpha_1^{0/0}$ mice, contrasting with the changes observed in the cerebellum of $\alpha_1^{0/0}$ mice and in the nRT of $\alpha_3^{0/0}$ mice (Kralic et al., 2006; Studer et al., 2006). Since VGAT is the vesicular transporter for GABA (and glycine), the storage of GABA in presynaptic vesicles remains operant and probably sustains tonic GABAergic inhibition in VB neurons.

Formation of potential GABA release sites at preserved GABAergic terminals

The loss of gephyrin clustering and GABAergic postsynaptic densities suggests that long-term maintenance of postsynaptic sites is not essential for GABAergic terminals. In the cerebellum, immunoelectron microscopy reveals that such "orphan" terminals make aberrant synapses with the spines of Purkinje cells (Fritschy and Panzanelli, 2006). Here, in VB neurons, GABA-positive terminals in the $\alpha_1^{0/0}$ mice are found connected to dendrites with electron dense structures resembling characteristic non-synaptic junctions regularly found in the thalamus (Lieberman and Spacek, 1997). This suggests that in absence of functional $GABA_A$ receptors, synapses are not stable and pre-synaptic terminals contact other structures made by postsynaptic cells, being spines in Purkinje cells or non-synaptic junctions in the VB. Whether the observed structures are equal to the common synaptic junctions remains speculative, as nothing is known about the protein content or function of them. Nevertheless, the accumulation of presynaptic vesicles to these sites suggest them to be the location of the transmitter release machinery. Strikingly, the morphology and therefore protein content of the presynaptic release zone changes drastically, suggesting an inductive mechanism from the postsynaptic cell.
In P30 $\alpha_1^{0/0}$ mice, gephyrin staining was found predominantly at small dendritic structures and almost never at synaptic glomeruli. Whether this is due to a higher protein-turnover rate in synaptic glomeruli or whether such small dendritic structures originate from different cells can not be elucidated due to the complex morphological arrangement in the thalamus. However, gephyrin staining was never found in dendrites innervated by presynaptic terminals which also displayed such non-synaptic junctions. We therefore conclude that there is no or only a short temporal overlay of these two specializations at a terminal. Whether disappearance of gephyrin facilitates the formation of such junctions or vice versa is not known.

Altogether we conclude that synaptic organization in a given brain region is highly dynamic and flexible. Upon a major impairments such as complete loss of the GABAergic postsynaptic specialization and activity, GABAergic presynaptic terminals are still capable of reorganizing intrinsic structures such that at least transmitter release and thus tonic inhibition remains functional.

3.6. Acknowledgments

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III. General Discussion

In this thesis, we investigated mechanisms underlying formation and maintenance of GABAergic synapses, with a special focus on clustering of PSD molecules. The following main results have been obtained:

(1) In cultured hippocampal cells, major postsynaptic proteins such as the $GABA_A$ receptor, gephyrin and NL2 cluster at synaptic sites, irrespective of the presence of GABAergic presynaptic input, suggesting a cell autonomous clustering mechanism

(2) The appearance of GABAergic postsynapses apposed to both, a GABAergic and a glutamatergic presynaptic terminal in striato-hippocampal co-cultures suggest that presynaptic terminals can be attracted by pre-formed postsynaptic protein aggregates, with a competitive advantage for GABAergic over glutamatergic terminals

(3) In Purkinje cells of collybistin-deficient mice, the lack of gephyrin clustering is constitutive. In these cells, targeting and accumulation of $GABA_A$ receptors and NL2 at GABAergic postsynapses takes place properly despite the absence of gephyrin

(4) In VB relay neurons of $\alpha_1^{0/0}$ mice, developmental loss of $GABA_A$ receptor expression from established GABAergic synapses leads to disruption of gephyrin clustering and loss of the whole GABAergic postsynaptic density, whereas presynaptic terminals are preserved

(5) GABAergic presynaptic terminals in adult VB neurons of $\alpha_1^{0/0}$ mice form specialized non-synaptic junctions with dendrites of relay neurons, constituting potential neurotransmitter release sites

Based on these findings, we conclude the following major points: First, the formation of synapses results from attraction of presynaptic terminals by preformed postsynaptic protein clusters to defined sites rather than vice versa; second, none of the proteins investigated so far is essential for clustering of postsynaptic proteins, but there is a hierarchy in their interaction; third, gephyrin is an important factor in regulating synapses

but is not at the top of the hierarchy, because its clustering depends on either collybistin or $GABA_A$ receptors; fourth, when GABAergic synapses are disrupted due to loss of $GABA_A$ receptors, presynaptic terminals are maintained but engage in non-synaptic contacts with target cells. Therefore, clustering of $GABA_A$ receptors and PSD molecules are essential for long-term maintenance of GABAergic synapses. We propose here a new model of how GABAergic synapses are formed and maintained (Figure 1).

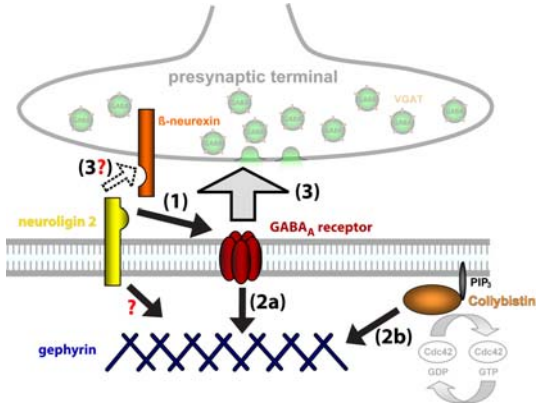

Figure 1

Postulated model of protein clustering hierarchy for the formation and maintenance of GABAergic synapses. (1) NL2 (or other cell adhesion molecules) marks the site of GABAergic synapse induction. Clustering of gephyrin highly depends on the $GABA_A$ receptor (2a) and – in Purkinje cells – collybistin (2b) expression. Preformed postsynaptic clusters are able to capture or stabilize presynaptic terminals, followed by formation of a synapse at this site (3). If this process occurs via NL2 or other cell adhesion molecules is not known.

Model of GABAergic synapse formation

In our proposed model of how GABAergic synapses form, clustering of postsynaptic molecules occurs in a cell autonomous manner and such preformed clusters then attract presynaptic terminals and trigger the formation of a synapse. We deduced a hierarchy in clustering of postsynaptic molecules based on the following findings: In cerebellar Purkinje cells of $\alpha_1^{0/0}$ mice, $GABA_A$ receptor expression is ablated at any developmental stage and gephyrin clustering is lost (Kralic et al., 2006). Nevertheless, as seen in electron microscopy, symmetric, presumably GABAergic synapses are able to form, but the number of symmetric synapses is significantly reduced compared to WT animals. Furthermore, many GABAergic presynaptic terminals form aberrant heterologous asymmetric synapses with spines (Fritschy et al., 2006). In these mice, NL2 is still able to cluster. Notably, NL2 does not cluster at such aberrant synapses, but is localized correctly at symmetric synapses on dendritic shafts (Patrizi et al., 2008). This finding suggests that NL2 is a key determinant for the localization of GABAergic synapses and is not misdirected to

alternative structures. Therefore, we attribute a role for NL2 (or other transsynaptic cell adhesion molecules) at the top of the hierarchy and speculate that NL2 contributes to determine sites of GABAergic synapse formation. Nevertheless, in NL2-deficient mice, GABAergic synapses are properly formed, showing that NL2 can be replaced in its role for synapse formation (Varoqueaux et al., 2006).

The second position for clustering GABAergic PSD proteins is attributed to the $GABA_A$ receptor. Upon its elimination in $\alpha_1^{0/0}$ mice, clustering of gephyrin is abolished and the number of synapses are significantly reduced in Purkinje cells (Fritschy et al., 2006) or even completely eliminated as seen in VB neurons (study 3). Furthermore, in vitro studies showed that the expression levels of functional postsynaptic receptors influence the rate of synapse formation (Fang et al., 2006; Deng et al., 2007). Taken together we conclude that the GABAergic synapses can form in the absence of the $GABA_A$ receptor, but the number of synapses formed is reduced and PSDs are not complete in their molecular composition as gephyrin is not clustered in the absence of $GABA_A$ receptors.

The contribution of the scaffolding protein gephyrin to formation of GABAergic synapses and clustering of postsynaptic proteins is not completely clarified. Gephyrin clustering highly depends on the presence of the $GABA_A$ receptor (Study 3; (Kralic et al., 2006)), even though a direct interaction has been shown only for the α2-subunit of the $GABA_A$ receptor (Tretter et al., 2008). Furthermore, in vitro, clustering of gephyrin has been shown to be induced by the GEF collybistin (Kins et al., 2000; Grosskreutz et al., 2001). Nevertheless, in collybistin-deficient mice, the absence of collybistin has differential, cell-type specific effects on gephyrin clustering. Thus, in Purkinje cells and hippocampal pyramidal cells of collybistin-deficient mice, gephyrin clustering is ablated whereas in other cell types such as cerebellar granule cells, gephyrin clustering occurs to a normal extent similar to WT animals (study 2; (Papadopoulos et al., 2007)). Whether other GEFs compensate for the loss of collybistin is not known. Down regulation or ablation of gephyrin in cultured neurons leads to a decrease of GABAergic synapses (Kneussel et al., 1999; Levi et al., 2004; Yu et al., 2007), whereas mutations of selective gephyrin sub-domains have been reported to increase the number of synapses (Lardi-Studler et al., 2007), suggesting a direct influence of gephyrin onto GABAergic postsynaptic clusters. Whether this is due to its interaction with collybistin which in turn activates several signaling cascades through Cdc42 activation is not known and requires further

investigation. Nevertheless, loss of gephyrin clustering in Purkinje cells of collybistin-deficient mice does not affect clustering of the $GABA_A$ receptor and NL2 and the formation of GABAergic synapses (study 2). We therefore conclude that gephyrin influences clustering of postsynaptic elements, but at a low hierarchy. We attribute an importance of gephyrin rather in fine-tuning mechanisms such as regulation of cluster size and stability, than synapse formation. Therefore, further studies focusing on gephyrin interaction partners as well as posttranslational modification of gephyrin will be necessary to better understand its roles at the GABAergic synapse.

In hippocampal primary cultured neurons, gephyrin, NL2 and the $GABA_A$ receptor are found in triple positive clusters together apposed to GABAergic and glutamatergic terminals, representing matched and mismatched synapses, respectively (study 1). The appearance of the three molecules together irrespective of the type of presynaptic input strongly argues that assembly of postsynaptic molecules is a cell autonomous process occurring independently of transmitter-related presynaptic cues. Furthermore, in striato-hippocampal co-cultures, a fraction of postsynaptic clusters facing a GABAergic and glutamatergic terminal at the same time strongly suggests that already formed postsynaptic clusters attract presynaptic terminals to form a synapse onto them, with a competitive advantage of GABAergic terminals over glutamatergic terminals (see discussion of study 1). The contact is likely to occur through cell adhesion molecule interaction. Whether this is mediated via NL2-neurexin interaction or through other cell adhesion molecules is not known. Considering its synaptogenic activity as well as the capability to define sites of GABAergic synapses, NL2 displays a likely candidate to attract presynaptic terminals. Investigating the effect of differential regulation of NL2 onto formation of such double-innervated clusters in striato-hippocampal cultures by up- and down regulation of NL2 would further elucidate the involvement of NL2 in these processes.

Maintenance and stability of GABAergic synapses

Once postsynaptic proteins have clustered and presynaptic specialization has formed, synapse maturation takes place (Craig et al., 2006). During this step, the size of the active zone and the PSD are regulated. This is reflected in the larger size of gephyrin clusters at correctly matched synapses compared to mismatched synapses and is likely to occur in a

transmitter release dependent manner (study 1). In line with the assumption that synapse maturation depends on neurotransmitter mediated receptor activation, non-functional synapses remain instable over time. Mismatched synapses, which are most probably complete in their postsynaptic composition are eliminated, even though elimination takes several days and is not a rapid process in the time-range of cellular protein turnover. In VB neurons of $\alpha_1^{0/0}$ mice GABAergic synapses are properly formed. Upon developmental loss of $GABA_A$ receptor expression, gephyrin clustering disappears and the PSDs are eliminated (study 3). This observation strongly suggests that the $GABA_A$ receptor is crucial for long-term maintenance of GABAergic synapses. Similar to the elimination of mismatched synapses, disruption of gephyrin clusters and disassembly of synapses does not occur rapidly suggesting a certain stability for these complexes. The elimination of non-functional synapses at later stages of synapse maturation might be due to fine-tuning mechanisms, possibly involving molecules activating signaling cascades such as collybistin. Therefore, deeper investigation of regulation and binding partners of proteins, which have shown to contribute to synapse stability such as gephyrin will lead to better understanding of mechanisms, how synapses are regulated and maintained.

Formation of non-synaptic junctions

In VB neurons of adult $\alpha_1^{0/0}$ mice, upon loss of the GABAergic PSDs, presynaptic terminals are preserved (study 3). Tonic inhibition remains unaltered in these mice (Peden et al., 2008), suggesting that the release of GABA by synaptic vesicles is maintained despite the disappearance of GABAergic synapses. At the ultrastructural level, we regularly observed GABA-positive terminals in $\alpha_1^{0/0}$ mice contacting relay cell dendrites at specialized non-synaptic junctions, which distinctly resembled "filamentous contacts": In WT animals, such contacts represent characteristic junctions between glutamatergic afferent terminals and VB relay neuronal dendrites in synaptic glomeruli and are highly abundant. They also occur between GABAergic terminals and VB neuron dendrites, though less frequently (Lieberman and Spacek, 1997). A major difference of non-synaptic junctions in the $\alpha_1^{0/0}$ mice compared to these "filamentous contacts" is the presence of presynaptic vesicles close to the contact, suggesting that they mediate neurotransmitter release (study 3). Strikingly, the morphology and therefore protein content of the presynaptic release zone

at these junctions is profoundly altered in comparison to the active zone of GABAergic synapses. We therefore interpret these observation that non-synaptic junctions in VB neurons of $\alpha_1^{0/0}$ mice still have an active zone containing proteins of the vesicle fusion machinery, and acquire in addition the proteins of the filamentous junction, possible under the influence of an inductive mechanism from the postsynaptic cell. This view is in line with our hypothesis that postsynaptic structures play a determinant role for attracting and stabilizing axon terminals onto them.

The formation of mismatched pre- and postsynaptic structures is not unique to this brain area. In Purkinje cells of $\alpha_1^{0/0}$ mice, the loss of GABAergic postsynapses leads to the formation of heterologous synapses with asymmetric postsynaptic densities at dendritic spines (Fritschy et al., 2006), and in low density hippocampal cultured neurons, GABAergic PSDs attract the formation of glutamatergic presynaptic terminals onto them (Brunig et al., 2002b). We speculate here that various dendritic trans-cellular structures can attract the formation of orphan protein aggregates, possibly through the trans-cellular binding of cell adhesion molecules. However, to substantiate this hypothesis, we would first need to demonstrate that proteins of the synaptic vesicle release machinery, such as SNAP25, are still present in these non-synaptic junctions.

However, the appearance of such aberrant junctions among pre- and postsynaptic elements argue for a binding competition among cell adhesion molecules. Many types of junctions incorporate common cell adhesion molecules such as molecules of the integrin and cadherin families, sharing homologies in their binding domains (Shapiro et al., 2007). Binding with the proper interaction partner likely results in the most stable complex but upon lacking binding partners, other "orphan" complexes appear to be stabilized – at least long enough to be detected. This is also reflected in the long-term stability of mismatched synapses in striato-hippocampal co-cultures, even when hippocampal cells receive abundant GABAergic presynaptic input.

All together, in this thesis we provide evidence that there is no essential protein required to form a GABAergic synapse, but clustering of postsynaptic proteins at GABAergic synapses follow a hierarchy. Furthermore, we showed here that the $GABA_A$ receptor is not crucial for the formation of GABAergic synapses, but a guarantor for their long-term stability. Elimination of the $GABA_A$ receptor leads to destabilization of the PSD. Nevertheless, orphan presynaptic structures are attracted by other trans-cellular protein

structures, ensuring a high flexibility of the synapses and making the system highly adaptable. Further, we suggest here that postsynaptic mechanisms display the dominant factors ensuring the formation of synapses at defined sites.

In view of the apparent lack of major phenotype in $\alpha_1^{0/0}$ mice due to a disruption of the thalamocortical system, the formation of non-synaptic junctions, while "aberrant", might represent a compensatory mechanism sufficient to maintain functional integrity despite the loss of synaptic GABAergic transmission.

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Abbreviations

aCSF	artificial cerebrospinal fluid
AIS	axon initial segment
CaMKII	Ca^{2+}/calmodulin dependent kinase II
CCD	charged chamber device
Cdc42	cell division cycle 42
DH	Dbl homology domain
DIV	days-*in-vitro*
DMEM	Dulbecco's Modified Eagel Medium
E	embryonic day
EGFP	enhanced green fluorescent protein
EPSP	excitatory postsynaptic potential
FCS	fetal calf serum
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
GAD	glutamic acid decarboxylase
GAD67-EGFP	EGFP-expression under the promoter of GAD
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GKAP	guanylate kinase associated protein
Glut	glutamate
GRIP	glutamate receptor interacting protein
GTP	guanosine triphosphate
HSA	human serum albumin
I	interneuron
IPSP	inhibitory postsynaptic potential
KCC2	K^+-Cl^- cotransporter 2
LI	lightly innervated
mIPSC	miniature inhibitory postsynaptic current
ML	molecular layer

Moco	molybdenum cofactor
NGS	normal goat serum
NI	not innervated
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter 1
NL2	neuroligin2
nRT	reticular nucleus (nucleus reticularis)
P	postnatal day
PB	phosphate buffer
PBS	phosphate buffered saline
PC	principal cell
PCL	Purkinje cell layer
PCR	polymerase chain reaction
PH	pleckstrin homology domain
PIP_3	phosphatidyl inositol triphosphate
PSD	postsynaptic density
PSD95	postsynaptic density protein of 95 kD
PT	presynaptic terminal
RhoGEF	catalytic GDP/GTP exchange domain of collybistin
SH3	*src* homology 3 domain
SI	strongly innervated
SNAP25	synaptosome-associated protein of 25 kD
SynCAM	synaptic cell adhesion molecule
SynGAP	synaptic GTPase activating protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing Triton X-100
tSNARE	target synaptosome-associated protein receptor
VB	ventrobasal complex
vGluT	vesicular glutamate transporter
VIAAT	vesicular inhibitory amino acid transporter
vSNARE	vesicle synaptosome-associated protein receptor
WT	wildtype

Curriculum Vitae

Personal:

Surname:	Petitjean
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Education:

2004 – present	PhD thesis: Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland Supervision: Prof. Dr. Jean-Marc Fritschy Title: Formation and Maintenance of GABAergic synapses Affiliation to the PhD program in Neuroscience of the Center for Neuroscience Zurich (ZNZ)
2006	Bordeaux PENS Training Center: European Synapse Summer School
1998 – 2003	Study in Biochemistry at the University of Zurich, Zurich, Switzerland Degree: Dipl. Biochem. Mat. Nat. Diploma thesis: Institute of Biochemistry, University of Zurich, Zurich, Switzerland Supervision: Prof. Dr. Peter Sonderegger, Dr. Sergio Gloor Title: Generation of Stable NG108 Clones Overexpressing Neurotrypsin
1994 – 1998	Kantonsschule Baden, AG, Switzerland Matura Type C (type science), 1998

Publications

Original articles

Lardi-Studler B, Smolinsky B, **Petitjean CM**, Koenig F, Sidler C,.Meier JC Fritschy JM, Schwarz G (2007) *Vertebrate-specific sequences in the gephyrin E-domain regulate cytosolic aggregation and postsynaptic clustering.* J Cell Sci, 120 (8), 1371-82

Petitjean CM, Peden DR, Herd MB, Durakoglugil MS, Rosahl TW, Wafford K, Homanics GE, Belelli D, Fritschy JM, Lambert JJ (2008) *Developmental maturation of synaptic and extrasynaptic $GABA_A$ receptors in mouse thalamic ventrobasal neurons.* J Physiol, 586 (4), 965-87

Petitjean CM, Bürli T, Sidler C, Fritschy JM (2009) *Postsynaptic mechanisms influence the formation of GABAergic synapses in hippocampal cultured neurons*. (submitted to Neuroscience)

Petitjean CM, Papadopoulos T, Varoqueaux F, Brose N, Betz H, Fritschy JM (2009) *Assembly of $GABA_A$ receptors and neuroligin2 in developing GABAergic synapses on cerebellar Purkinje cells is gephyrin-independent in collybistin-knockout mice*. (in preparation)

Abstracts

Petitjean CM, Sidler C, Fritschy JM (2006) *Postsynaptic mechanisms influence the formation of GABAergic synapses in a striato-hippocampal co-culture system.* Swiss Society of Neuroscience (SSN) annual meeting, Basel BS, Switzerland

Petitjean CM, Sidler C, Fritschy JM (2006) *Postsynaptic mechanisms influence the formation of GABAergic synapses in a striato-hippocampal co-culture system*. 5th Forum of European Neuroscience (FENS) Meeting, Vienna, Austria

Petitjean CM, Fritschy JM (2007) *Influence of $GABA_A$ receptor subunit expression changes on synapse maintenance in the thalamus of developing $\alpha_1^{0/0}$ mice*. Swiss Society of Neuroscience (SSN) annual meeting, Bern BE, Switzerland

Petitjean CM, Herd MB, Peden DR, Homanics GE, Lambert JJ, Belelli D, Fritschy JM (2007) *Influence of $GABA_A$ receptor ($GABA_AR$) subunit expression changes on synapse maturation in the thalamus of developing $\alpha_1^{0/0}$ mice*. Gephyrin meeting, Chebrex, VD, Switzerland

Petitjean CM, Herd MB, Peden DR, Homanics GE, Lambert JJ, Belelli D, Fritschy JM (2007) *Influence of $GABA_A$ receptor ($GABA_AR$) subunit expression changes on synapse maturation in the thalamus of developing $\alpha_1^{0/0}$ mice.* Annual Meeting of the Society for Neuroscience (SfN), San Diego, CA, USA

Petitjean CM, Papadopoulos T, Varoqueaux F, Brose N, Betz H, Fritschy JM (2008) *Assembly of $GABA_A$ receptors and neuroligin2 in developing GABAergic synapses of the cerebellar Purkinje cells in collybistin knockout mice is gephyrin-independent*. 6th Forum of European Neuroscience (FENS) Meeting, Geneva, Switzerland

Seminar

Petitjean CM (2008) *Doing a PhD project at the University of Zurich*. V Jornadas de Jóvenes Investigadores del Campus Biosanitario de Albacete, Spain

Appendix: original article

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Developmental maturation of synaptic and extrasynaptic $GABA_A$ receptors in mouse thalamic ventrobasal neurones

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Thalamic ventrobasal (VB) relay neurones express multiple $GABA_A$ receptor subtypes mediating phasic and tonic inhibition. During postnatal development, marked changes in subunit expression occur, presumably reflecting changes in functional properties of neuronal networks. The aims of this study were to characterize the properties of synaptic and extrasynaptic $GABA_A$ receptors of developing VB neurones and investigate the role of the α_1 subunit during maturation of GABA-ergic transmission, using electrophysiology and immunohistochemistry in wild type (WT) and $\alpha_1^{0/0}$ mice and mice engineered to express diazepam-insensitive receptors (α_{1H101R}, α_{2H101R}). In immature brain, rapid (P8/9–P10/11) developmental change to mIPSC kinetics and increased expression of extrasynaptic receptors (P8–27) formed by the α_4 and δ subunit occurred independently of the α_1 subunit. Subsequently ($\geq$ P15), synaptic α_2 subunit/gephyrin clusters of WT VB neurones were replaced by those containing the α_1 subunit. Surprisingly, in $\alpha_1^{0/0}$ VB neurones the frequency of mIPSCs decreased between P12 and P27, because the α_2 subunit also disappeared from these cells. The loss of synaptic $GABA_A$ receptors led to a delayed disruption of gephyrin clusters. Despite these alterations, GABA-ergic terminals were preserved, perhaps maintaining tonic inhibition. These results demonstrate that maturation of synaptic and extrasynaptic $GABA_A$ receptors in VB follows a developmental programme independent of the α_1 subunit. Changes to synaptic $GABA_A$ receptor function and the increased expression of extrasynaptic $GABA_A$ receptors represent two distinct mechanisms for fine-tuning GABA-ergic control of thalamic relay neurone activity during development.

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$GABA_A$ receptors mediate fast GABA-ergic neurotransmission, and are assembled from a large family of subunits (Barnard *et al.* 1998). $GABA_A$ receptors differing in subunit composition are distinguished by their function, pharmacology, subcellular localization (e.g. synaptic *versus* extrasynaptic) and spatio-temporal expression patterns (Fritschy & Brünig, 2003; Korpi & Sinkkonen, 2006). This heterogeneity is exemplified in the adult rodent thalamus, which contains at least three major $GABA_A$ receptor populations (Wisden *et al.* 1992; Fritschy & Mohler, 1995; Zhang *et al.* 1997). In relay nuclei, such as VB, or the lateral geniculate nucleus (LGN), synaptic ($\alpha_1\beta_2\gamma_2$) and extrasynaptic ($\alpha_4\beta_2\delta$) $GABA_A$ receptors coexist, whereas the reticular nucleus (nRT) and intralaminar nuclei mainly express synaptic $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors. These receptor populations have defined functional and pharmacological properties, with α_1- and α_3-$GABA_A$ receptors being diazepam sensitive and mediating phasic inhibition, whereas $\alpha_4\delta$-$GABA_A$ receptors are diazepam insensitive, highly sensitive to neurosteroids and 4,5,6,7- tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol (THIP) and mediate tonic inhibition (Sur *et al.* 1999; Browne *et al.* 2001; Belelli *et al.* 2005; Jia *et al.* 2005; Farrant & Nusser, 2005; Chandra *et al.* 2006; Bright *et al.* 2007). At the network level, these distinct $GABA_A$ receptors contribute to the regulation of thalamocortical rhythmic activity associated with sleep, wakefulness and vigilance and are implicated in seizure disorders (Huntsman & Huguenard, 2000; Sohal *et al.* 2003).

D. R. Peden, C. M. Petitjean and M. B. Herd contributed equally to this work.

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The $GABA_A$ receptor subtypes of relay nuclei have a delayed expression during postnatal development. In rodents at birth, expression of α_1, α_4, β_2 and δ subunits is low and relay neurones in the VB and LGN mainly express α_2 and β_3 subunits (Laurie *et al.* 1992; Fritschy *et al.* 1994). An apparent switch in expression occurs during brain maturation, with α_1- gradually replacing α_2-$GABA_A$ receptors, a re-organization proposed to influence the kinetics of IPSCs (Okada *et al.* 2000). In contrast, in the nRT, α_3-$GABA_A$ receptors are abundant at every postnatal age examined, although a transient expression of the α_5 subunit around P7 has been reported (Studer *et al.* 2006). The presence of multiple $GABA_A$ receptor subtypes within single neurones (α_2- in immature and α_1- and α_4-$GABA_A$ receptors in mature relay neurones) raises the question of how their assembly and subcellular targeting are regulated, and to what extent their function is determined by subunit composition. Thus, developing VB neurones represent an excellent model to address these fundamental questions.

Here, we investigated the changes to $GABA_A$ receptors in VB neurones during postnatal ontogeny using electrophysiological and morphological approaches in wild-type (WT), α_1 subunit null ($\alpha_1^{0/0}$) β_2 subunit null ($\beta_2^{0/0}$) and 'knock-in' α_{1H101R} and α_{2H101R} mice (McKernan *et al.* 2000; Sur *et al.* 2001; Vicini *et al.* 2001). Importantly, the developmental change to mIPSC kinetics (which plays a critical role in thalamo-cortical oscillations) and the establishment of extrasynaptic receptors, occurred independently of the α_1 subunit. Unexpectedly, the mIPSCs of $\alpha_1^{0/0}$ VB neurones disappeared during maturation, indicating that the α_2 subunit was not replaced. Therefore, we used immunohistochemistry for $GABA_A$ receptor subunits and pre- and postsynaptic markers of GABA-ergic transmission to determine how this loss of function affects GABA-ergic synapses in VB neurones of $\alpha_1^{0/0}$ mice. Intriguingly, the disruption of synaptic transmission caused by deletion of the α_1 subunit had no effect on extrasynaptic receptor expression, which increased with development (P8–27) similarly for VB neurones derived from WT and $\alpha_1^{0/0}$ mice.

Methods

All experiments have been approved by the local authorities and were performed in accordance with European Community Council Directive 86/609/EEC and with the institutional guidelines of the Universities of Zurich and Dundee.

Thalamic slice preparation and electrophysiology

The α_{1H101R}, α_{2H101R}, $\beta_2^{0/0}$ and $\alpha_1^{0/0}$ mice utilized for electrophysiological experiments were generated on a mixed C57BL6–129SvEv background at the Merck Sharp & Dohme Research Laboratories at the Neuroscience Research Centre in Harlow as previously described (McKernan *et al.* 2000; Sur *et al.* 2001). Experiments were conducted on slices prepared from the first two generations of WT, α_{1H101R}, α_{2H101R}, $\beta_2^{0/0}$ and $\alpha_1^{0/0}$ breeding pairs derived from the corresponding heterozygous $^{+/0}$ mice bred at the University of Dundee.

Thalamic slices were prepared from mice of either sex (P8–27) according to standard protocols (Belelli *et al.* 2005). Animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The brain was rapidly removed and placed in oxygenated 'ice cold' maintenance solution containing (mM): 225 sucrose, 2.95 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 D-glucose (pH of 7.4; 330–340 mosmol l^{-1}). The tissue was maintained in this 'ice-cold' solution whilst horizontal 300–400 μm slices were cut using a Vibratome (St Louis, MO, USA). The slices were incubated at 32°C for 1 h in an oxygenated, extracellular solution (ECS) containing (mM): 126 NaCl, 2.95 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-glucose and 2 $MgCl_2$ (pH 7.4; 300–310 mosmol l^{-1}). Subsequently, slices were maintained at room temperature (20–23°C) before being used for recordings. Whole-cell patch clamp recordings were performed at 35°C from thalamic nucleus reticularis (nRT) and VB neurones visually identified with an Olympus BX51 microscope (Olympus, Southall, UK) equipped with DIC/IR optics as previously described (Belelli *et al.* 2005). Patch pipettes were prepared from thick walled borosilicate glass (Garner Glass Co., Claremont, CA, USA) and had open tip resistances of 3–5 MΩ when filled with an intracellular solution that contained (mM): 140 CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 1 $CaCl_2$, 5 QX-314 (pH 7.3 with CsOH, 300–305 mosmol l^{-1}). Miniature inhibitory postsynaptic currents (mIPSCs) were recorded using an Axopatch 1D or 200B amplifier (Axon Instruments, Union City, CA, USA) at a holding potential of −60 mV in ECS that, additionally contained 2 mM kynurenic acid (Sigma-Aldrich, UK) and 0.5 μM tetrodotoxin (TTX; Tocris Bioscience, Bristol, UK) to block ionotropic glutamate receptors and sodium-dependent action potentials, respectively. Series resistance and whole-cell capacitance were estimated by cancelling the fast current transients evoked at the onset and offset of brief (10–20 ms) 3–5 mV voltage-command steps. Series resistance ranged between 5 and 16 MΩ. Series resistance compensation of 60–80% was employed in the presence of lag values of 10–20 μs. Recordings were discarded if the series resistance changed (10% tolerance) during the course of the experiment. Currents were filtered at 2 kHz using an 8-pole low pass Bessel filter and recorded on to digital audio tape using a DTR 1205 recorder for subsequent offline analysis.

Drugs. THIP (gaboxadol, 10^{-2} M), bicuculline methobromide (10^{-2} M), strychnine (10^{-3} M) and nipecotic acid (10^{-1} M) were dissolved in water, whereas zolpidem was prepared as a concentrated (1000×) stock solution in DMSO. These stock solutions were diluted in ECS to the desired concentration. The final maximum DMSO concentration (0.1%v/v) had no effect on mIPSCs, or the tonic current. All modulatory agents were applied *via* the perfusion system (2–4 ml min^{-1}) and allowed to infiltrate the slice for a minimum of 10 min while recordings were acquired. With the exception of THIP, which was a generous gift of Bjarke Ebert (H. Lundbeck A/S, Copenhagen Valby, Denmark), and TP003 (Merck Sharp & Dohme – see Dias *et al.* 2005) all drugs tested were obtained from either Sigma-Aldrich, UK, or Tocris Bioscience (Bristol, UK).

Data analysis. Data was analysed offline using the Strathclyde Electrophysiology Software, WinEDR/WinWCP (J. Dempster, University of Strathclyde, UK). Individual mIPSCs were detected using a −4 pA amplitude threshold detection algorithm and visually inspected for validity. Accepted events were analysed for peak amplitude, 10–90% rise time, charge transfer and time for events to decay from peak by 90% (T_{90}). To minimize the contribution of dendritically generated currents, which are subject to cable filtering, analysis was restricted to events with a rise time ≤ 1 ms. A minimum of 100 accepted events per cell were digitally averaged by alignment at the mid-point of the rising phase, and the mIPSC decay fitted by either monoexponential ($y(t) = Ae^{(-t/\tau)}$), or biexponential ($y(t) = A_1e^{(-t/\tau_1)} + A_2e^{(-t/\tau_2)}$) functions using the least squares method, where A is amplitude, t is time and τ is the decay time constant. Analysis of the S.D. of residuals and use of the F test to compare goodness of fit revealed that the average mIPSC decay was always best fitted with the sum of two exponential components. Thus, a weighted decay time constant (τ_w) was also calculated according to the equation:

$$\tau_w = \tau_1 P_1 + \tau_2 P_2,$$

where τ_1 and τ_2 are the decay time constants of the first and second exponential functions and P_1 and P_2 are the proportions of the synaptic current decay described by each component.

The mIPSC frequency was determined over 10 s bins for 2 min with the EDR program using a detection method based on the rate of rise of events (with a minimum rate of rise of 35–40 pA ms^{-1}) and visual scrutiny. The tonic current was calculated as the difference between the holding current before and after application of bicuculline methobromide (30 μM; Belelli *et al.* 2005). We previously demonstrated the effects of bicuculline in this respect to be reproduced by 100 μM picrotoxin, a structurally distinct $GABA_A$ receptor antagonist (Belelli *et al.* 2005). In order to compare the efficacy of tonic and phasic inhibition per unit of time (s), the phasic charge was calculated by multiplying the individual mIPSC charge by the number of mIPSCs per second (effectively the frequency).

All results are reported as the arithmetic mean ± standard error of the mean (S.E.M.). Statistical significance of mean data was assessed with Student's t test, paired or unpaired as appropriate, or byregular or repeated measures (RM) ANOVA followed *post hoc* by the Newman–Keul's test as appropriate, using the SigmaStat software package (Systat Software Inc., San Jose, CA, USA). The large sample approximation of the Kolmogorov–Smirnov (KS) test (SPSS Inc., Chicago, IL, USA) was used to compare the distribution of individual mIPSC parameters. For a stringent comparison, the level of significance was set at $P < 0.01$ for KS tests.

Immunohistochemistry

All morphological experiments were performed on WT and $\alpha_1^{0/0}$ mice generated on a mixed C57BL/6J–129Sv/SvJ at the University of Pittsburgh (Vicini *et al.* 2001) and obtained from heterozygous ($\alpha_1^{+/0}$) breeding pairs, or from first generation WT and $\alpha_1^{0/0}$ breeding pairs derived from $\alpha 1^{+/0}$ mice. Four different sets of experiments were performed using the following antibodies: guinea pig antisera against $GABA_A$ receptor subunits α_1, α_2 and α_3 and γ_2 (raised in-house); rabbit antibodies against the α_4 (PhosphoSolutions, Aurora, CO, USA; cat. no. 844-GA4N) and δ subunit (Chemicon International, Temecula, CA, USA; cat. no. AB9752), vesicular GABA transporter (VGAT; Synaptic Systems, Göttingen, Germany, cat. no. 131003); mouse monoclonal antibody 7a against gephyrin (Synaptic Systems, cat. no. 147011). The characterization of antibodies was based on previous results (Kralic *et al.* 2006; Studer *et al.* 2006) and on the known regional and cellular expression pattern of their epitope.

Immunoperoxidase staining. The distribution of $GABA_A$ receptor subunits in the thalamus was compared in WT and $\alpha_1^{0/0}$ mice during postnatal development (P10, P20, P30, P60, $n = 3$ per age and genotype) in sections processed for immunoperoxidase staining. Mice were deeply anaesthetized with pentobarbital (Nembutal, 50 mg kg^{-1}, I.P.) and transcardially perfused with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.15 M phosphate buffer, pH 7.4. The brains were extracted immediately after the perfusion and postfixed in the same solution (P10, 24–36 h; P20, 12 h; P30 and adult, 4–6 h). Tissue was then processed for antigen retrieval as described (Kralic *et al.* 2006), cryoprotected with 30% sucrose in phosphate-buffered saline (PBS), and cut parasagitally at 40 μm from frozen tissue with a sliding microtome.

Sections were collected in PBS and stored in antifreeze solution (15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) prior to use.

Sections were incubated free-floating overnight at 4°C with primary antibodies against $GABA_A$ receptor subunits (α_1, 1 : 20 000; α_2, α_4, δ, affinity purified, 1–2 μg ml^{-1}; α_3, γ_2, 1 : 3000) in Tris buffer containing 2% normal goat serum and 0.2% Triton X-100. Sections were then washed and incubated for 30 min at room temperature with biotinylated secondary antibodies (1 : 300; Jackson Immunoresearch, West Grove, PA, USA), followed by incubation in avidin–biotin complex (1 : 100 in Tris buffer) for 30 min (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA), washed again and finally reacted with diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO, USA) in Tris buffer (pH 7.7) containing 0.015% hydrogen peroxide. The colour reaction was stopped after 5–15 min with 'ice-cold' PBS. Sections were then mounted on gelatin-coated slides and air-dried. Finally, they were dehydrated with ethanol, cleared in xylol, and coverslipped with Eukitt (Erne Chemie, Dällikon, Switzerland). Sections from WT and $\alpha_1^{0/0}$ mice were processed in parallel under identical conditions to minimize variability in staining intensity.

Immunofluorescence staining. Colocalization of $GABA_A$ receptor α_1, α_2, α_4 and γ_2 subunit with gephyrin at presumptive postsynaptic sites was analysed in WT and $\alpha_1^{0/0}$ mice using double immunofluorescence staining in sections prepared from fresh-frozen tissue. Mice (P10–12, P15, P20, P30; $n = 3$–5 per age and per genotype) were anaesthetized with isoflurane and decapitated, and the brain was extracted rapidly and frozen with powdered dry ice. Transverse sections were cut at 12 μm with a cryostat, mounted onto gelatin-coated glass slides, air dried at room temperature for 1 min, and stored at –20°C. They were then thawed at room temperature and fixed in methanol at −20°C for 2 min. After two washes in PBS, the sections were incubated overnight at 4°C with a mixture of primary antibodies diluted in PBS containing 4% normal goat serum. Sections were washed extensively in PBS and incubated for 30 min at room temperature with the corresponding secondary antibodies conjugated to Cy3 (1 : 500, Jackson Immunoresearch), or Alexa488 (1 : 1000, Molecular Probes, Eugene, OR, USA). Sections were washed again with PBS and coverslipped with mounting medium (DAKO, Carpinteria, CA, USA).

For covisualization of presynaptic terminals and gephyrin clusters in WT and $\alpha_1^{0/0}$ mice (P30, P60, $n = 3$ per age and genotype), tissue was prepared as described (Schneider Gasser *et al.* 2006). Mice were decapitated as above, the brain rapidly removed and placed in oxygenated 'ice-cold' artificial cerebrospinal fluid (aCSF). Slices were prepared with a vibrating microtome and incubated for 20 min in aCSF at 34°C. They were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, extensively washed and incubated overnight in 30% sucrose in PBS for cryoprotection. Transverse sections (16 μm) were cut from frozen slices, mounted onto gelatin-coated slides, air dried at room temperature for 1 min, and stored at −20°C. They were then processed for double immunofluorescence staining with antibodies to VGAT (1 : 3000) and gephyrin (1 : 1000) as described above.

For quantification of GABA-ergic terminals in the VB of WT and $\alpha_1^{0/0}$ mice (P10, P30, P60, $n = 3$ per age and genotype), immunofluorescence staining was performed with anti-VGAT antibodies in perfusion-fixed tissue that was not processed for antigen retrieval (see immunoperoxidase staining, above). After overnight incubation in primary antibodies and washing, free-floating sections were incubated with secondary antibodies conjugated to Cy3 in Tris buffer containing 2% normal goat serum for 30 min at room temperature, washed and coverslipped with DAKO mounting medium.

Image analysis. Sections processed for immunoperoxidase staining were visualized with brightfield microscopy (Axioplan; Zeiss) using the mosaic software (Explora Nova, La Rochelle, France) for image acquisition. For each antibody, similar acquisition parameters were selected for WT and $\alpha_1^{0/0}$ animals. Images were cropped to the desired dimensions in Adobe Photoshop CS. Minimal adjustments of contrast and brightness were made to entire images, if needed.

Double-immunofluorescence staining was visualized by confocal microscopy (Zeiss LSM-510 Meta; Jena, Germany) using a 100× Plan-Apochromat objective (N.A. 1.4). The pinhole was set to 1 Airy unit for each channel and separate colour channels were acquired sequentially. The acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. For display, images were processed with the image-analysis program Imaris (Bitplane; Zurich, Switzerland). Images from both channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. A low-pass 'edge preserving' filter was used for images displaying α_1, α_2, or VGAT staining.

Postsynaptic clustering of gephyrin and gephyrin–α subunit colocalization was quantified from single confocal sections (512 × 512 pixels) at a magnification of 90 nm pixel^{-1} in 8 bit grayscale images, using a threshold segmentation algorithm (minimal intensity, 90–130; size 0.08–0.8 μm^2). From the gephyrin–α subunit colocalized images, all structures > 0.057 μm^2 were counted, representing a minimum of 70% overlay of both channels to be considered as colocalized (ImageJ imaging software, NIH, Bethesda, MD, USA). The data were quantified in six sections per animal ($n = 3$ per genotype and age, Kruskal–Wallis followed by Dunn's

multiple comparison test; Prism, GraphPad Software, San Diego, CA, USA).

For quantification of the density of GABA-ergic presynaptic terminals, volumes were reconstructed from stacks of 13 confocal images (512 × 512 pixels) spaced by 0.25 μm at a magnification of 90 nm pixel^{-1}. All files were blinded and individual terminals were identified as isolated objects with 1 μm^3 minimal apparent volume and counted automatically (Imaris; Bitplane). Size distribution of VGAT positive terminals was analysed in single confocal sections (ImageJ imaging software). These data were quantified in six sections per animal ($n = 3$ per genotype and age, Kolmogorov–Smirnov test).

Results

Developmental changes in the properties of VB synaptic receptors

We investigated in detail the influence of development (P8–9; P10–11; P12–14; P15–22 and P23–27) of the mouse on the amplitude, decay and frequency of VB mIPSCs (Fig. 1; Table 1). In agreement with a previous study on rat VB neurones (Huntsman & Huguenard, 2000), these mIPSC parameters exhibited significant changes across the five developmental stages ($P < 0.05$, one-way ANOVA; see Table 1). For P8–9 neurones the mIPSCs were of relatively large amplitude (-99 ± 4.0 pA; $n = 33$), decayed relatively slowly ($\tau_W = 9.0 \pm 0.4$ ms; $n = 33$) and occurred at a frequency of 8.5 ± 0.8 Hz ($n = 33$). However, within the next 24–48 h (P10–11) the mIPSC characteristics changed considerably. In particular, the mIPSC decay time (τ_W) decreased (5.4 ± 0.3 ms; $n = 18$, $P < 0.05$ *versus* P8–9, Newman–Keul's test) and with subsequent development was further reduced (P12–14, 5.1 ± 0.3 ms, $n = 24$; P15–22, 3.9 ± 0.1 ms, $n = 66$; P23–27, 3.4 ± 0.2 ms, $n = 18$, all $P < 0.05$ *versus* P8–9). The change to the mIPSC decay that occurred between P8–9 and P10–11 was not accompanied by an alteration to the mIPSC amplitude (P8–9, -99 ± 4 pA, $n = 33$; P10–11, -96 ± 8 pA; $n = 18$, $P > 0.05$ *versus* P8–9). However, by P12–14 the mIPSC amplitude had decreased (-68 ± 3 pA; $n = 24$, $P < 0.05$ *versus* P8–9), but subsequently remained stable with further development (P15–22, -74 ± 2 pA, $n = 66$; P23–27, -79 ± 4 pA, $n = 18$, $P > 0.05$ *versus* P12–14). Hence, given the developmental changes to amplitude and duration, the charge passed by each mIPSC was significantly greater at P8–9 (897 ± 46 fC; $n = 33$), than for later time points (e.g. P23–27, 287 ± 20 fC, $n = 18$, $P < 0.05$). The frequency of mIPSCs increased significantly throughout the developmental period (e.g. P8–9, 8.5 ± 0.8 Hz, $n = 33$; P15–22, 26 ± 2.6 Hz, $n = 66$; $P < 0.05$, one-way ANOVA), with this effect becoming significantly different from P8–9 by P15–22 (Fig. 1, Table 1). Although the phasic charge per event is significantly reduced with development, the increase in mIPSC frequency with maturation results in the total phasic charge per second not being significantly changed during the developmental period studied here ($P > 0.05$, one-way ANOVA, Table 1).

By contrast, the amplitude of mIPSCs recorded from mouse nRT neurones was little influenced by the developmental stage of the animal (P8–9, -67 ± 6 pA, $n = 8$; P15–22, -64 ± 3 pA, $n = 21$; $P > 0.05$, unpaired Student's t test). However, early in development the decay of nRT mIPSCs was significantly slower (P8–9, 21.4 ± 0.7 ms, $n = 8$; P15–22, 15.8 ± 0.9 ms, $n = 21$; $P < 0.001$; unpaired t test).

The α_1 subunit is expressed synaptically in P15–22 VB neurones

The relatively fast mIPSC decay ≥ P15 is consistent with the synaptic expression of the α_1 subunit, a view supported by both immunohistochemistry and electrophysiological studies with zolpidem (Belelli *et al.* 2005; Kralic *et al.* 2006). However, although low nanomolar concentrations of zolpidem are selective for receptors containing both α_1 and γ_2 subunits, higher concentrations enhance the function of the equivalent receptors incorporating α_2, or α_3 subunits (Rudolph & Mohler, 2006). Therefore, to confirm that synaptic $GABA_A$ receptors of P15–22 VB neurones contain the α_1 subunit, we utilized the α_{1H101R} 'knock-in' mouse (McKernan *et al.* 2000). Receptors incorporating this mutant α_1 subunit are insensitive to diazepam and zolpidem (Rudolph & Mohler, 2006). The mIPSCs (amplitude = -74 ± 4 pA; $\tau_W = 3.7 \pm 0.2$ ms, $n = 29$, $P > 0.05$ *versus* WT, unpaired t test) recorded from VB neurones of P15–22 α_{1H101R} mice were indistinguishable from their WT counterparts, demonstrating that this genetic manipulation does not influence $GABA_A$ receptor expression, or function in these neurones (Fig. 2*A*). A concentration (100 nM) of zolpidem that should be relatively selective for α_1 subunit containing receptors and that produced a clear prolongation of WT mIPSCs, was ineffective for VB mIPSCs of α_{1H101R} mice (10 ± 4% increase, $n = 6$, $P > 0.05$; cf. WT 57 ± 7% increase, $n = 6$, $P < 0.001$; one-way RM ANOVA, Figs 2*A* and *B*), although, at the less selective concentration of 1 μM, zolpidem did produce a modest, yet significant prolongation of the VB mIPSC decay (α_{1H101R}, 20 ± 4% increase of τ_w, $n = 6$; WT, 73 ± 14% increase, $n = 6$; $P < 0.01$ for both strains, one-way RM ANOVA Fig. 2*B*). However, the effect of 1 μM zolpidem on the mIPSC τ_W of VB neurones of WT mice was significantly greater than that for α_{1H101R} mice ($P < 0.001$, two-way RM ANOVA).

The anxiolytic TP003 is functionally selective for recombinant receptors containing γ_2 and α_3 subunits compared with the equivalent receptors incorporating the α_1 subunit (Dias *et al.* 2005). TP003 (100 nM)

prolonged the decay of mIPSCs recorded from WT nRT neurones (44 ± 12% increase, $n = 6$, $P < 0.05$, one-way RM ANOVA), but was significantly less effective in prolonging the decay of WT VB mIPSCs (20 ± 4% increase, $n = 5$, $P < 0.05$ *versus* WT, two-way RM ANOVA; see Fig. 2*B*). Hence, collectively these data are consistent with VB neurones expressing synaptic $GABA_A$ receptors incorporating the α_1 subunit, although at this age

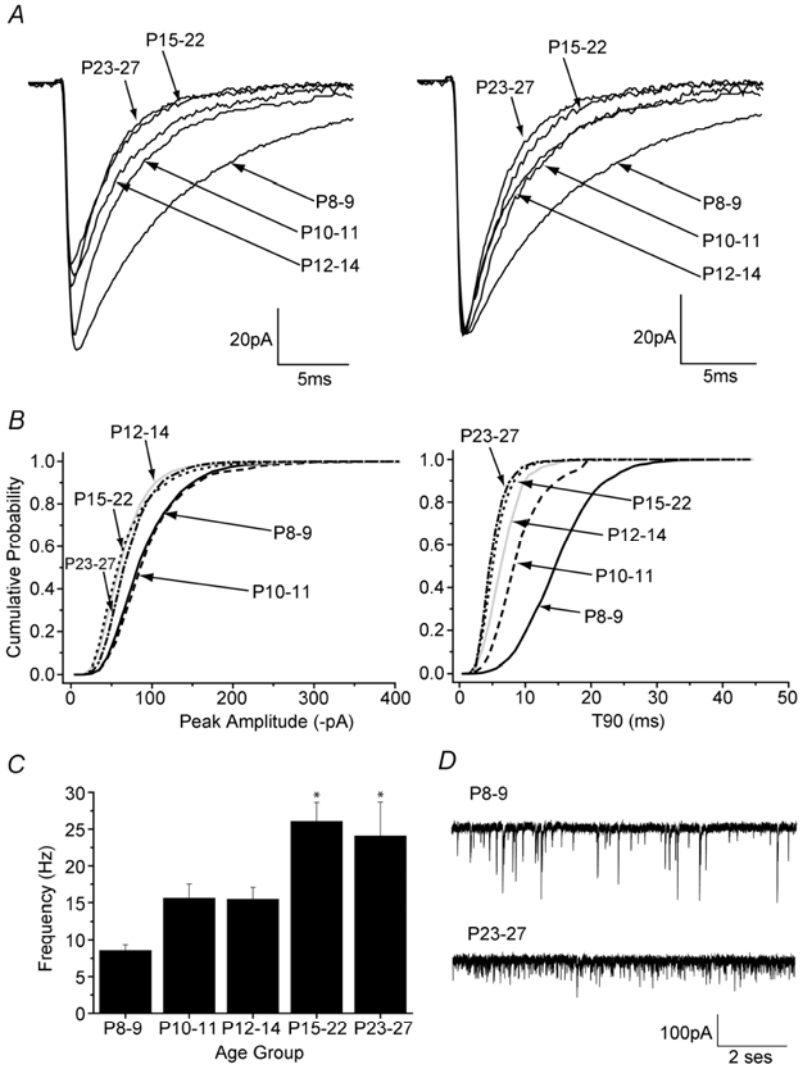


Figure 1. The properties of mIPSCs recorded from VB neurones of WT mice are developmentally regulated

A, superimposed ensemble averages of mIPSCs recorded from exemplar VB neurones of WT mice at five different developmental stages (P8–9, P10–11, P12–14, P15–22 and P23–27). The traces illustrate the changes to peak amplitude (left panel) and synaptic current decay (right panel) that occur with development. In the right-hand panel, all traces are normalized to the peak amplitude of the exemplar P23–27 recording to highlight the change to the mIPSC decay that occurs with maturation. *B*, combined cumulative probability plots of the peak amplitude (left panel) and of the time required for individual mIPSCs to decay from peak amplitude to 10% of peak (T_{90}, right panel). The plots are constructed from peak amplitude and T_{90} values obtained from 945 to 2142 mIPSCs collected from 10 representative neurones for each of the five age groups investigated. Note the progressive leftward shift of both the peak amplitude and T_{90} values with development ($P < 0.01$; KS test), although the changes to amplitude and kinetics are not synchronous. *C*, bar graph illustrating the increase of VB mIPSC frequency with development. Data were obtained from 18–66 cells. Error bars indicate the S.E.M. *D*, recordings obtained from exemplar WT VB neurones for the P8–9 (top trace) and P23–27 (bottom trace) age groups illustrating the increase of mIPSC frequency with development (*$P < 0.05$, one-way ANOVA).

Table 1. Summary of the developmental changes (P8–27) to $GABA_A$ receptor-mediated phasic and tonic transmission of wild type VB neurones

	P8-9	P10-11	P12-14	P15-22	P23-27
mIPSC amplitude (pA)	99 ± 4 (n = 33)	96 ± 8 (n = 18)	68 ± 3 (n = 24)	74 ± 2 (n = 66)	79 ± 4 (n = 18)
mIPSC τ_w (ms)	9 ± 0.4	5.4 ± 0.3	5.1 ± 0.3	3.9 ± 0.1	3.4 ± 0.2
mIPSC charge transfer (−fC)	897 ± 46	533 ± 53	351 ± 24	295 ± 12	287 ± 20
mIPSC frequency (Hz)	8.5 ± 0.8	15.6 ± 2.0	15.4 ± 1.7	26.0 ± 2.6	24.0 ± 4.7 (n = 16)
Total phasic charge (−fC)	7389 ± 789	8070 ± 1346	5129 ± 576	8159 ± 1013	7000 ± 1384
Tonic current (−pA)	23 ± 6 (n = 11)	30 ± 9 (n = 7)	37 ± 6 (n = 8)	74 ± 8 (n = 25)	93 ± 15 (n = 9)
Tonic charge (−fC)	23 198 ± 6003	30 416 ± 8894	36 509 ± 6446	74 174 ± 8400	93 304 ± 14,822
Total charge (−fC)	28 264 ± 5499*	38 238 ± 9,562*	41 499 ± 6828*	79 309 ± 8492*	98 177 ± 14,779*
THIP-induced current (−pA)	80 ± 23 (n = 6)	ND	ND	293 ± 34 (n = 7)	337 ± 18 (n = 4)
Nipecotic acid-induced current (−pA)	105 ± 31 (n = 6)	ND	ND	415 ± 88 (n = 5)	ND
Tonic/total charge (%)	73 ± 8*	76 ± 5*	87 ± 3*	92 ± 1*	94 ± 1*

Note that all individual mIPSC properties (peak amplitude, τ_w, charge transfer and frequency), amplitude of the tonic current together with the relative contribution (%) of the tonic to the total charge and the THIP-induced current are significantly different across the different developmental stages investigated here ($P < 0.05$, ANOVA). Similarly, the nipecotic-induced current is significantly greater at P15–22 than at P8–9 ($P < 0.001$ unpaired t test). The total phasic charge does not exhibit significant changes across development ($P > 0.05$, ANOVA). ND, not determined; *values derived only from the sample of cells for which a paired (i.e. matched from the same cell) estimation of the phasic and tonic charge was performed.

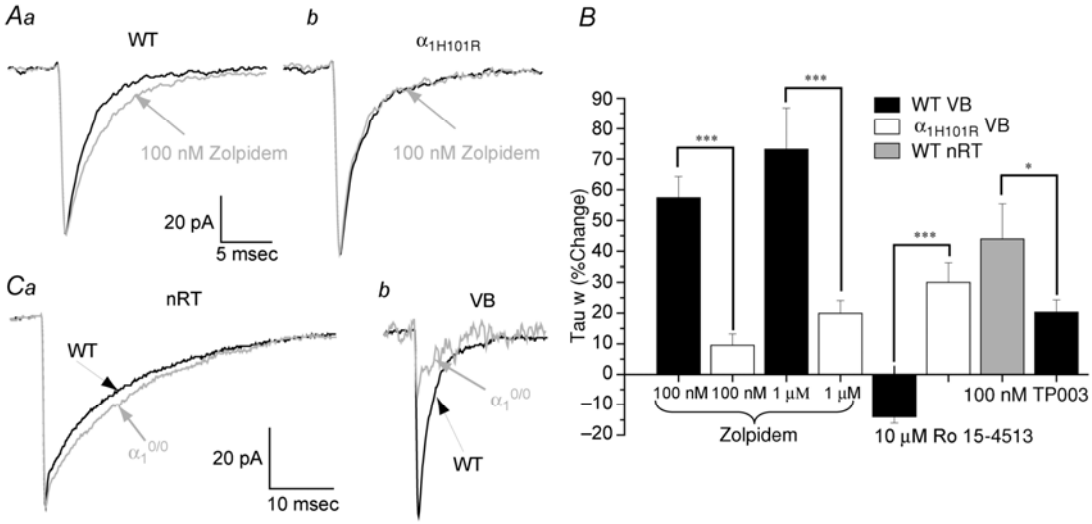


Figure 2. The selective synaptic expression of the $GABA_A$ receptor α_1 subunit in P15–22 VB neurones
A, superimposed ensemble averages of VB mIPSCs recorded in the absence (black traces) and presence of the α_1 subunit selective imidazopyridine, zolpidem (100 nM, grey traces) from exemplar WT (*Aa*) and α_{1H101R} (*Ab*) neurones. Note that zolpidem prolongs the decay of WT mIPSCs, but not those recorded from α_{1H101R} mice, consistent with the synaptic expression of the α_1 subunit. *B*, summary bar graph illustrating the effects of zolpidem (100 nM, 1 μM) and Ro15-4513 (10 μM) upon the decay (τ_w, expressed as percentage change) of mIPSCs recorded from VB neurones of WT (black bars) and α_{1H101R} (open bars) mice. Also illustrated are the effects of the α_3 selective ligand, TP003 (100 nM), upon the decay of mIPSCs recorded from WT nRT (grey bar) and VB (black bar) neurones. *C*, superimposed representative mIPSC averages recorded from exemplar WT (black traces) and $\alpha_1{}^{0/0}$ (grey traces) nRT (*Ca*) and VB (*Cb*) neurones. Deletion of the α_1 subunit causes a clear reduction in VB mIPSC frequency (not shown) and a decrease in amplitude of the remaining synaptic currents. Note that the majority (32/44 neurones, i.e. 73%) of P15–22 $\alpha_1{}^{0/0}$ neurones were silent and no mIPSCs were evident for P23–27 $\alpha_1{}^{0/0}$ neurones (16 cells tested). By contrast, deletion of the α_1 subunit had no significant effect on the mIPSCs recorded from nRT neurones. Data were obtained from 3–6 cells. Asterisks in *B* indicate statistical differences (*$P < 0.05$, ***$P < 0.001$). Error bars indicate S.E.M.

(P15–22), there may be minor populations of receptors incorporating α_2 or α_3 subunits.

In VB neurones the α_4 subunit is mainly associated with the δ subunit and located extrasynaptically (Sur *et al.* 1999; Peng *et al.* 2002; Chandra *et al.* 2006; Kralic *et al.* 2006). However, in thalamus, immunoprecipitation studies suggest an additional coassembly of the α_4 and γ_2 subunits (Sur *et al.* 1999). To determine whether synaptic currents in WT VB neurones are mediated in part by receptors incorporating both α_4 and γ_2 subunits, we made use of the pharmacological profile of Ro15-4513. This ligand acts as a partial agonist of recombinant receptors incorporating these subunits, whereas it displays inverse agonist action at equivalent receptors containing α_1, α_2, α_3, or α_5 subunits (Benson *et al.* 1998). Consistent with synaptic receptors containing α_1 and γ_2 subunits Ro15-4513 (10 μM) produced a modest acceleration of the mIPSC decay ($14 \pm 2\%$ decrease of τ_W, $n = 3$, $P < 0.05$, one-way RM ANOVA see Fig. 2*B*) in WT VB neurones. Furthermore, it significantly prolonged the mIPSC decay of α_{1H101R} VB neurones ($30 \pm 6\%$ increase of τ_W, $n = 5$, $P < 0.01$, one-way RM ANOVA; Fig. 2*B*), in line with its partial agonist effect of receptors containing an arginine residue in position 101. The contrasting effects of Ro15-4513 on the phasic currents of WT and α_{1H101R} VB neurones further confirms the expression of synaptic α_1 and γ_2 subunits, and additionally suggests that synaptic receptors incorporating α_4 and γ_2 subunits are not expressed in these P15–22 VB neurones.

The expression of the α_1 subunit in VB neurones is developmentally regulated: studies with the $\alpha_1^{0/0}$ mouse

In agreement with the conclusions above, deletion of the α_1 subunit greatly compromised inhibitory synaptic transmission in P23–27 VB neurones, with none of the cells sampled ($n = 16$) exhibiting mIPSCs (Fig. 3). In stark contrast, for P8–9 and P10–11 VB neurones, this genetic manipulation had no significant effect on the frequency, amplitude, or kinetics of mIPSCs when compared with aged matched WT controls ($P > 0.05$, unpaired t test Figs 3 and 4, Tables 1 and 2), suggesting the synaptic incorporation of the α_1 subunit occurs during neuronal

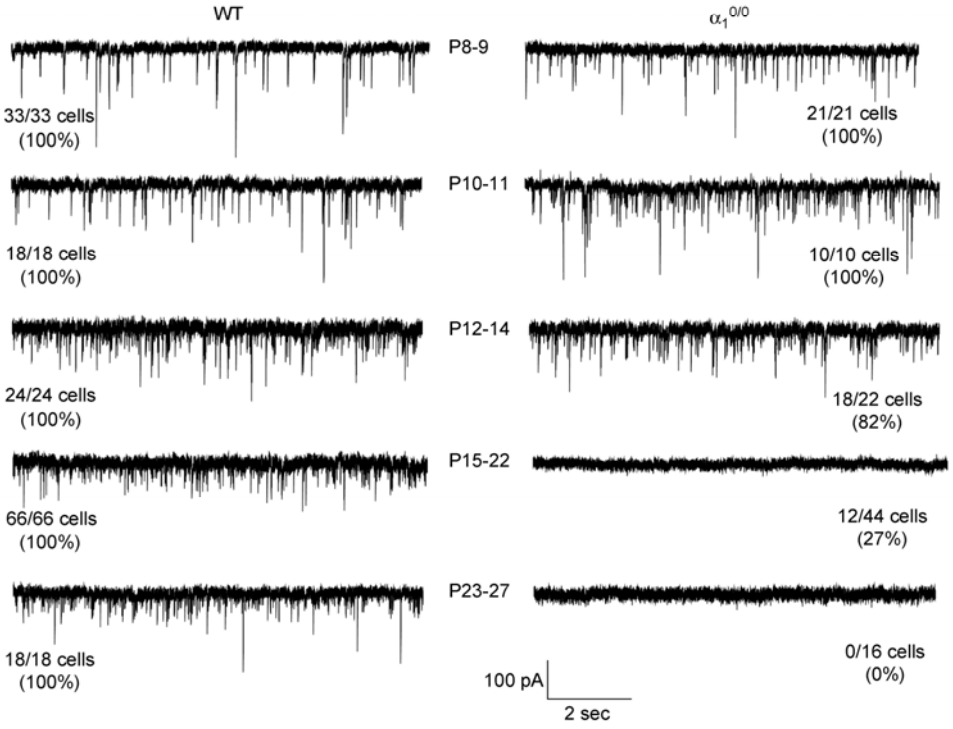


Figure 3. $GABA_A$ receptor-mediated synaptic transmission is disrupted by deletion of the α_1 subunit, but only later in development
Recordings obtained from exemplar WT (left-hand column) and $\alpha_1^{0/0}$ (right-hand column) VB neurones for five different age groups. Early in development (P8–11) mIPSCs recorded from VB neurones of $\alpha_1^{0/0}$ mice are indistinguishable from those of WT. However, beyond P14, the majority of VB neurones from $\alpha_1^{0/0}$ mice are devoid of mIPSCs. The proportion of recorded cells displaying synaptic currents is stated below each exemplar trace.

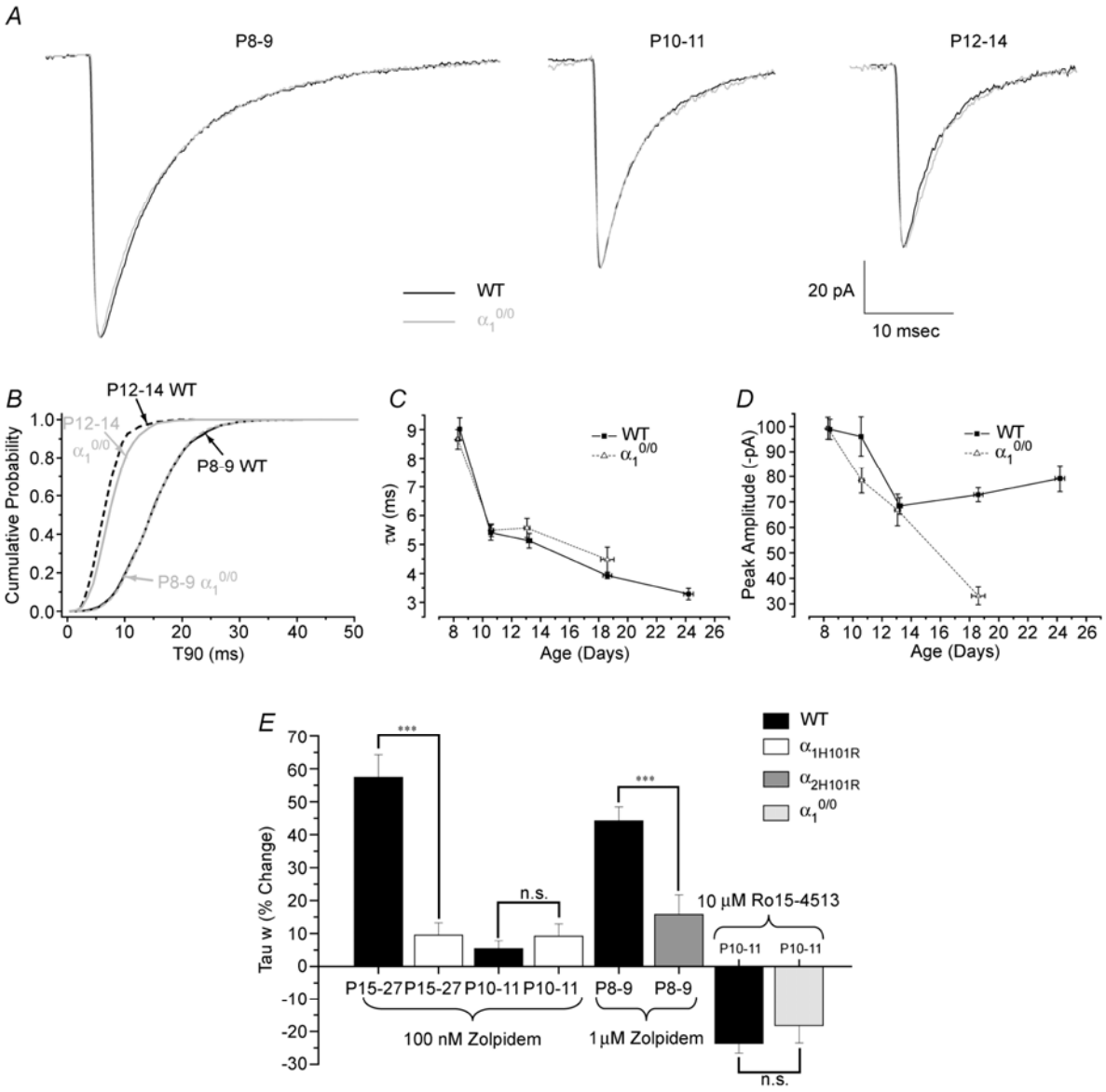


Figure 4. The developmental change (P8–14) to mIPSC amplitude and decay do not require the $GABA_A$ α_1 subunit

A, representative superimposed averages of mIPSCs recorded from exemplar WT (black traces) and $\alpha_1^{0/0}$ (grey traces) VB neurones of P8–9, P10–11 and P12–14 mice. Note that, even in the absence of the α_1 subunit, the mIPSC decay shortens at P10–11 and P12–14 compared with P8–9. *B*, a combined cumulative probability plot of the mIPSC T_{90} values obtained from 774–2142 events collected from 10 representative VB neurones of WT and $\alpha_1^{0/0}$ VB neurones at P8–9 and P12–14 (black curves, WT; grey curves, $\alpha_1^{0/0}$). The leftward shift in T_{90} values observed in VB neurones of P12–14 $\alpha_1^{0/0}$ mice indicates that synaptic expression of the α_1 subunit is not a prerequisite for the shortening of the mIPSC decay that occurs in the second postnatal week. *C* and *D*, graphs illustrating the influence of development (P8–27) on the τ_W (*C*) and the peak amplitude (*D*) of mIPSCs recorded from VB neurones of WT (■) and $\alpha_1^{0/0}$ (△) mice. *X*-axis values are presented as the mean age of each developmental group studied. Data were obtained from 18–66 cells. *E*, summary bar graph illustrating the effects of zolpidem (100 nM, 1 μM) and Ro15-4513 (10 μM) upon the decay (τ_W, expressed as percentage change) of mIPSCs recorded from VB neurones of WT (black bars), α_{1H101R} (open bars), α_{2H101R} (dark grey bars) and $\alpha_1^{0/0}$ (light grey bars) mice. Data were obtained from 4–7 neurones. Error bars indicate S.E.M. Note that for the different developmental stages the standard error associated with each age group is also illustrated in *C* and *D*.

Table 2. Summary of the developmental changes (P8–27) to $GABA_A$ receptor-mediated phasic and tonic transmission of $\alpha_1^{0/0}$ VB neurones

	P8-9	P10-11	P12-14†	P15-22§	P23-27‡
mIPSC amplitude (−pA)	99 ± 4 (n = 21)	79 ± 5 (n = 10)	67 ± 6 (n = 18)	36 ± 4 (n = 12)	ND
mIPSC τ_W (ms)	8.7 ± 0.3	5.5 ± 0.2	5.6 ± 0.3	4.8 ± 0.4	ND
mIPSC charge transfer (−fC)	826 ± 49	439 ± 30	420 ± 50	194 ± 38	ND
mIPSC frequency (Hz)	7.5 ± 1.0	9.5 ± 2.2	10.6 ± 1.3	14.0 ± 2.8	ND
Total phasic charge (−fC)	6084 ± 908	4218 ± 1028	4849 ± 890	2033 ± 308	ND
Tonic current (−pA)	28 ± 8 (n = 11)	ND	ND	55 ± 7 (n = 21)	ND
Tonic charge (−fC)	27 674 ± 7670	ND	ND	54 974 ± 7241	ND
Total charge (−fC)	33 390 ± 8288*	ND	ND	55 470 ± 7304**	ND
Tonic/total charge (%)	76 ± 7*	ND	ND	99 ± 0.4**	ND

§Not including the 32 cells of 44 cells tested (73%) that were silent. †Not including the 4 cells of the 22 cells tested (18%) that were silent (note the 4 silent cells were all derived from day 14 animals). ‡No mIPSCs in all (16) cells tested. ND, not determined. *Values derived only from the sample of cells (n = 11) for which a paired (i.e. matched from the same cell) estimation of the phasic and tonic charge was performed. **Value derived from all the cells (n = 21) exhibiting a tonic conductance including those without any detectable mIPSCs. Note that the tonic contribution to the total charge was significantly greater at **P15-22** *versus* **P8-9** ($P < 0.05$).

maturation. This transition develops during the second and third postnatal week as 18% (4 of 22) of P12–14 and 73% (32 of 44) of P15–22 VB neurones sampled from $\alpha_1^{0/0}$ mice were devoid of mIPSCs (Fig. 3, Tables 1 and 2). The specificity of the mutation for VB neurones was substantiated by recording mIPSCs of nRT neurones from $\alpha_1^{0/0}$ mice, which were indistinguishable from their WT counterparts (Fig. 2*C*). Synaptic $GABA_A$ receptors of ≥ P16 VB neurones incorporate a β_2 subunit (Belelli *et al.* 2005). However, mirroring the results with the $\alpha_1^{0/0}$ mice, in younger neurones, deletion of the β_2 subunit had no significant effect on VB mIPSCs (frequency = 9.8 ± 1 Hz; amplitude = −83 ± 6 pA; τ_W = 7.9 ± 0.5 ms, n = 12, $P > 0.05$ *versus* WT, unpaired t test). Collectively, these results suggest during maturation the synchronized synthesis of both α_1 and β_2 subunits to form synaptic $\alpha_1\beta_2\gamma_2$ receptors.

Does expression of the α_1 subunit produce the developmental change in mIPSC kinetics of VB neurones?

As described above, the mIPSC decay time reduces with development. Receptors incorporating the α_1 subunit are reported to have relatively fast kinetics compared with equivalent receptors containing the α_2 or α_3 subunit and are implicated in the appearance of rapidly decaying mIPSCs in developing CGCs and cortical neurones (Goldstein *et al.* 2002; Ortinski *et al.* 2004; Bosman *et al.* 2005; Belelli *et al.* 2006). Hence, given the differential impact of deleting the α_1 subunit on mIPSCs at P8–9 and P15–22, the change in mIPSC kinetics may be due to the increased expression of the α_1 subunit with development. Here, the mIPSC τ_W of VB neurones decreased substantially within a 24–48 h period between P8–9 and P10–11 (Table 1; Fig. 1). For WT P8–9 neurones, a non-selective concentration (1 μM) of zolpidem prolonged the decay of mIPSCs (τ_W = 44 ± 4% increase, n = 7, $P < 0.05$, one-way RM ANOVA), an effect significantly reduced for equivalent recordings made from P8–9 neurones derived from α_{2H101R} mice (τ_W = 16 ± 6% increase, n = 5, $P < 0.001$ *versus* WT, two-way RM ANOVA; Fig. 4*E*). These results confirm the presence of synaptic receptors containing the α_2 and γ_2 subunit at this developmental stage.

Should the increased expression of the α_1 subunit cause the change to the mIPSC kinetics that occurs within 24–48 h at P10–11, then deletion of the α_1 subunit (in common with P15–22 neurones) would have a substantial effect on the VB mIPSCs (cf. P8–9 synapses which mainly incorporate α_2-$GABA_A$ receptors). However, surprisingly mIPSCs were evident in all (n = 10) P10–11 VB neurones of $\alpha_1^{0/0}$ mice tested (Figs 3 and 4). In these $\alpha_1^{0/0}$ neurones neither the mIPSC amplitude (79 ± 5 pA) nor the frequency (9.5 ± 2.2 Hz) was significantly different from WT age matched controls ($P > 0.05$, unpaired t test; Fig. 4, Tables 1 and 2). Importantly, at this developmental stage mIPSCs recorded from $\alpha_1^{0/0}$ VB neurones exhibited a similarly rapid decay (τ_W = 5.5 ± 0.2 ms, n = 10) to WT mIPSCs (τ_W = 5.4 ± 0.3 ms, n = 18, $P > 0.05$, unpaired t test; see Fig. 4*C*, Tables 1 and 2). Furthermore, even by P12–14, mIPSCs were still evident in the majority of $\alpha_1^{0/0}$ VB neurones (82%, i.e. 18 out of 22 cells tested – note the 4 'silent' cells were from P14 mice; see Fig. 3). Such mIPSCs were of a similar amplitude ($\alpha_1^{0/0}$, 67 ± 6 pA, n = 18; WT, 68 ± 3 pA, n = 24; $P > 0.05$, unpaired t test) and time course ($\alpha_1^{0/0}$: τ_W = 5.6 ± 0.3 ms, n = 18; WT: τ_W = 5.1 ± 0.3 ms, n = 24; $P > 0.05$, unpaired t test) to

their WT counterparts (Fig. 4*A*–*C*). By P15–22 the majority of $\alpha_1^{0/0}$ VB neurones were 'silent' (only 12 cells exhibited mIPSCs from 44 neurones tested). For those neurones exhibiting mIPSCs, the events were of reduced amplitude ($\alpha_1^{0/0}$, 36 ± 3.7 pA, $n = 12$; WT, 74 ± 2.5 pA, $n = 66$; $P < 0.001$, unpaired t test; see Figs 2*C* and 4*D*). The mean mIPSC frequency, although reduced, was not significantly different from that of WT neurones ($\alpha_1^{0/0}$, 14 ± 2.8 Hz, $n = 12$; WT, 26 ± 2.6 Hz, $n = 63$; $P > 0.05$, unpaired t test – note this does not take into account the majority of $\alpha_1^{0/0}$ VB neurones which were 'silent'). Hence, the disappearance of synaptic $GABA_A$ receptors from the VB neurones of $\alpha_1^{0/0}$ mice does not occur in an 'all or nothing' manner.

As described above, 100 nM zolpidem produced a substantial prolongation of mIPSCs recorded from WT but not α_{1H101R} VB P15–22 neurones. However, this concentration of the α_1 subunit selective ligand had little effect on the decay of mIPSCs of P10–11 neurones derived from either WT or α_{1H101R} mice (WT, $5 \pm 2\%$, $P > 0.05$; α_{1H101R}, $9 \pm 4\%$, $P > 0.05$; one-way RM ANOVA; WT *versus* α_{1H101R}, $P > 0.05$, two-way RM ANOVA, Fig. 4*E*). Therefore, although compensatory changes may occur as a consequence of the deletion of the α_1 subunit, importantly the collective results of these experiments clearly establish that the developmental change to mIPSC kinetics can occur in the absence of the α_1 subunit. However, it is conceivable that the subsequent further decrease of the mIPSC decay that occurs post P12 (Tables 1 and 2) may be due to the synaptic incorporation of the α_1 subunit. Indeed, the decay of the remaining mIPSCs of P15–22 $\alpha_1^{0/0}$ neurones (presumably mediated by residual α_2-$GABA_A$ receptors) was significantly prolonged, compared to their WT counterparts ($\alpha_1^{0/0}$ $\tau_W = 4.8 \pm 0.4$ ms, $n = 12$; WT $\tau_W = 3.9 \pm 0.1$ ms; $n = 66$; $P < 0.01$, unpaired t test).

Theoretically the fast mIPSCs evident for P10–11 neurones could be mediated by synaptic receptors incorporating the α_4 subunit as it is expressed in VB neurones and recombinant receptors containing α_4 and γ_2 subunits are known to be associated with relatively rapid kinetics (Lagrange *et al.* 2007; Picton & Fisher, 2007). As described above, Ro15-4513 acts as a positive allosteric modulator of receptors incorporating α_4 and γ_2 subunits and a partial inverse agonist of the equivalent receptors incorporating α_{1-3}, or α_5 subunits. However, this compound did not prolong but produced a modest shortening of the mIPSC decay of both WT and $\alpha_1^{0/0}$ VB P10–11 neurones, with no significant differences between genotypes (WT = $24 \pm 3\%$ decrease, $P < 0.01$; $\alpha_1^{0/0} = 18 \pm 5\%$ decrease, $P < 0.05$, one-way RM ANOVA; WT *versus* $\alpha_1^{0/0}$ $P > 0.05$, two-way RM ANOVA; Fig. 4*E*). Hence, it is unlikely that the brief mIPSCs, characteristic of this developmental stage are mediated by α_4-$GABA_A$ receptors.

Developmental changes in the properties of VB extrasynaptic receptors

We have previously reported the presence of a large 'tonic' current in P16–24 mouse VB neurones, which is mediated by extrasynaptic $GABA_A$ receptors (Belelli *et al.* 2005). Given the changes to the synaptic $GABA_A$ receptors of VB neurones, we investigated whether their extrasynaptic receptors were similarly subject to developmental plasticity. Such extrasynaptic receptors contain the δ subunit (Porcello *et al.* 2003) and in the rat thalamus, early in postnatal development, the δ subunit mRNA levels are relatively low (Laurie *et al.* 1992). In agreement, application of bicuculline (30 μM) to P8–9 neurones produced an outward current of only 23 ± 6 pA ($n = 11$; see Fig. 5*A*, Table 1), a response considerably less than that reported for P16–24 neurones (Belelli *et al.* 2005). We therefore determined the tonic current at different developmental stages (Fig. 5, Table 1). The tonic current amplitude exhibited significant changes across the five developmental stages ($P < 0.05$, one-way ANOVA; see Table 1). Thus, although the magnitude of the tonic current was modestly augmented at P10–11 and at P12–14 (30 ± 9 pA, $n = 7$; 37 ± 6 pA, $n = 8$, respectively; $P > 0.05$ *versus* P8–9 for either age group) it then increased substantially (P15–22, 74 ± 8 pA, $n = 25$; P23–27, 93 ± 15 pA, $n = 9$; $P < 0.005$ *versus* P8–9 for both age groups; Fig. 5), i.e. from P8–9 to P23–27 the tonic current increased in magnitude by ~4 fold. The relatively small tonic current at P8–9 may be a consequence of a variety of factors including changes to neuronal size, limited expression of extrasynaptic receptors, or perturbations to the local ambient GABA concentration.

The whole-cell capacitance of VB neurones did not change with development ($P > 0.05$, one-way ANOVA), suggesting little difference in the overall size of the neuronal cell soma (see Methods) and consequently the current density increases with maturation. For example, when normalized to whole-cell capacitance the tonic conductance of P8–9 VB neurones (16 ± 3.9 pS pF^{-1}, $n = 11$) was significantly less ($P < 0.001$ unpaired t test) than that of P23–27 neurones (59 ± 9.0 pS pF^{-1}; $n = 9$). However, our conclusion presumes all of the measured 'tonic' current to emanate from the cell body.

In the thalamus, THIP acts as a selective ligand for thalamocortical extrasynaptic $GABA_A$ receptors (Belelli *et al.* 2005; Cope *et al.* 2005; Jia *et al.* 2005; Chandra *et al.* 2006). As observed for the tonic current, the magnitude of the current induced by 1 μM THIP also increased significantly ($P < 0.05$, one-way ANOVA) with development (P8–9, -80 ± 23 pA; P15–22, -293 ± 34 pA; P23–27, -337 ± 18 pA; $n = 4$–7; see Fig. 6*A* and *C*, Table 1). However, relating these responses to their corresponding tonic current (i.e. induced by bicuculline alone) the THIP-induced current was ~3.5-fold that of the corresponding tonic current at both P8–9 and P23–27.

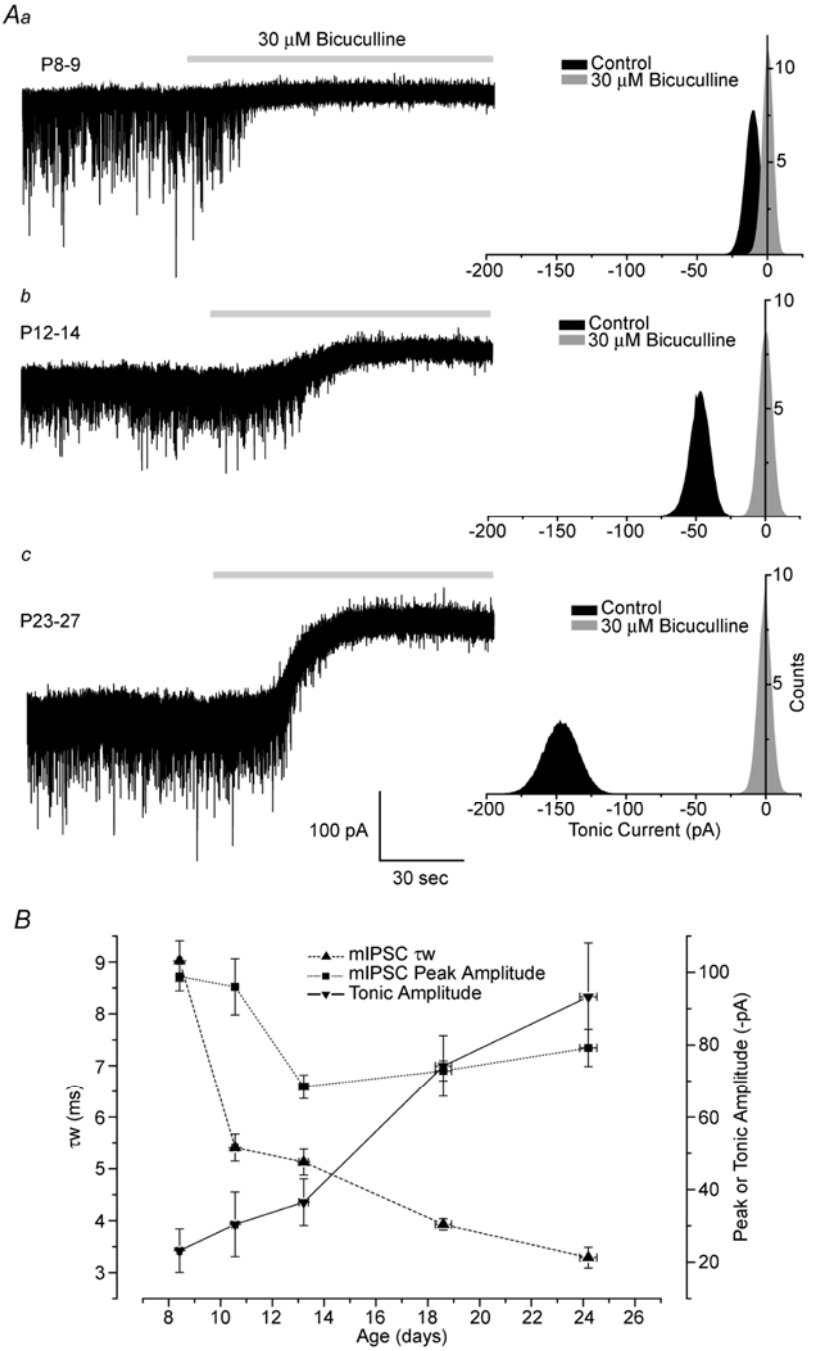
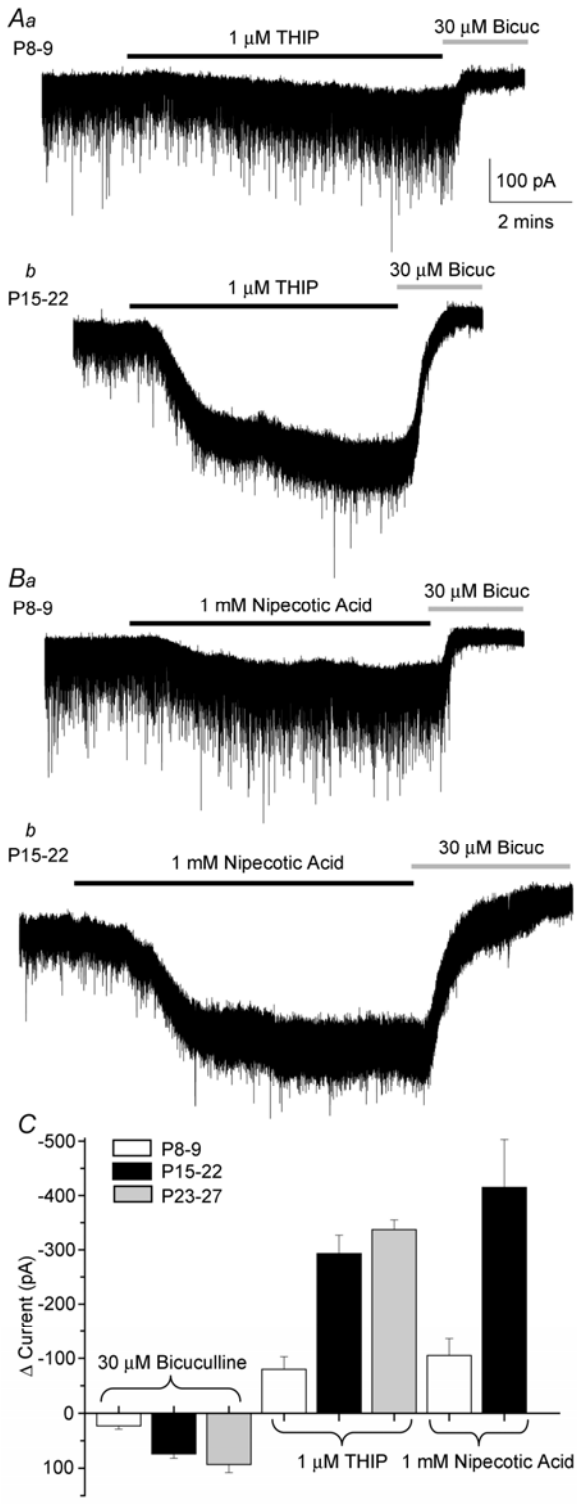


Figure 5. The tonic current of VB neurones increases with development
A, left panels, whole-cell recordings obtained from VB neurones of WT mice at P8–9 (*Aa*), P12–14 (*Ab*) and P23–27 (*Ac*). Right panels, the corresponding all-points histograms, normalized to the holding current recorded in the presence of bicuculline. Application of 30 μM bicuculline (grey) reveals a $GABA_A$ receptor-mediated tonic current, which increases in magnitude with development. *B*, a graph illustrating the developmental changes in mIPSC τ_w (left axis) and mIPSC peak, or tonic current amplitudes (right axis). *X*-axis values are presented as the mean age of each group studied. Note that the most dramatic changes to τ_w, peak amplitude and the magnitude of the tonic current occur at different developmental stages. Data were obtained from 18–66 cells for the mIPSCs and 7–25 cells for the tonic currents. Error bars indicate the S.E.M. Note that for the different developmental stages, the standard error associated with each age group is also illustrated in *B*.



In cerebellum, cerebral cortex and hippocampus, GABA transporters (GATs) influence both phasic and tonic inhibition (Nusser & Mody, 2002; Ortinski *et al.* 2006). Four GABA transporters have been identified and they are differentially distributed in the CNS (Conti *et al.* 2004). For P8–9 and P15–22 neurones the non-selective GAT inhibitor nipecotic acid (1 mM) induced an inward current of -105 ± 31 pA ($n = 6$) and -415 ± 88 pA ($n = 5$), respectively ($P < 0.001$, P15–22 *versus* P8–9, unpaired t test; see Fig. 6*B* and *C*, Table 1). Relating these responses to their corresponding tonic current demonstrated that nipecotic acid increased the tonic current by ~4.6- and 5.6-fold for P8–9 and P15–22 neurones, respectively.

Collectively, the experiments with THIP and nipecotic acid suggest that the developmental increase in the tonic conductance is primarily a consequence of an increase in extrasynaptic receptor expression.

α_1-$GABA_A$ receptors do not contribute to the tonic current of P15–22 VB neurones

Although in older neurones phasic inhibitory synaptic transmission was greatly compromised by deletion of the α_1 subunit, recordings from such VB $\alpha_1^{0/0}$ neurones revealed the characteristic membrane noise evident for WT neurones, suggesting the presence of a 'tonic' conductance (Fig. 7). In confirmation, bicuculline (30 μM) induced an outward current (55 ± 7 pA, $n = 21$) for P15–22 $\alpha_1^{0/0}$ VB neurones that was not significantly different from WT neurones ($P > 0.05$, unpaired t test; see Fig. 7). These data reveal that synaptic $GABA_A$ receptors of VB neurones can be eliminated by the deletion of the α_1 subunit, with little or no apparent perturbation of extrasynaptic $GABA_A$ receptor expression. This conclusion agrees with the finding that 'tonic inhibition' of VB neurones is abolished by deletion of the α_4 subunit (Chandra *et al.* 2006), suggesting little or no contribution of α_1-$GABA_A$ receptors to the tonic current.

Figure 6. An increase in extrasynaptic receptor expression primarily accounts for the developmental increase of the tonic conductance of VB neurones
A, the selective extrasynaptic $GABA_A$ receptor agonist THIP (gaboxadol, 1 μM) increases the magnitude of the tonic current in exemplar VB neurones of both P8–9 (*Aa*) and P15–22 (*Ab*) WT mice. However, note that the effect of the agonist is much greater for neurones from the older age group. *B*, the non-selective GAT inhibitor nipecotic acid (1 mM) increases the tonic current amplitude in exemplar VB neurones of both P8–9 (*Ba*) and P15–22 (*Bb*) WT mice. In common with THIP, the effect of nipecotic acid is greater for P15–22 than for P8–9 neurones. *C*, bar graph summarizing the change in holding current in response to the bath application of bicuculline (30 μM), THIP (1 μM) and nipecotic acid (1 mM) to WT VB neurones of P8–9 (open bars), P15–22 (black bars) and P23–27 (grey bars) mice. Data were obtained from 4 to 25 cells. Error bars indicate S.E.M.

Unaltered developmental maturation of the α_2 subunit in VB of $\alpha_1^{0/0}$ mice

Immunohistochemistry for $GABA_A$ receptor subunits was used to complement the analysis of the developmental changes in the properties of synaptic $GABA_A$ receptors in VB neurones. Mirroring electrophysiological findings, delayed expression of the α_1 was observed in WT mice. While being almost undetectable at P10, α_1 subunit immunoreactivity became prominent at P20 (Fig. 8*A* and *G*). In parallel, α_2 subunit staining was very strong at birth (not shown) and decreased gradually to disappear between P10 and P20 (Fig. 8*B* and *H*). These changes are in striking contrast to the α_3 subunit-immunoreactivity, which selectively labelled the nRT and remained rather constant throughout postnatal development (Fig. 8*C* and *I*). In $\alpha_1^{0/0}$ mice, the developmental maturation of the α_2 and α_3 subunit was the same as in WT (Fig. 8, middle and right column), and the absence of the α_1 subunit was not compensated for by these subunits. These observations are in line with the complete loss of synaptic $GABA_A$ receptor-mediated currents in VB neurones of $\alpha_1^{0/0}$ mice.

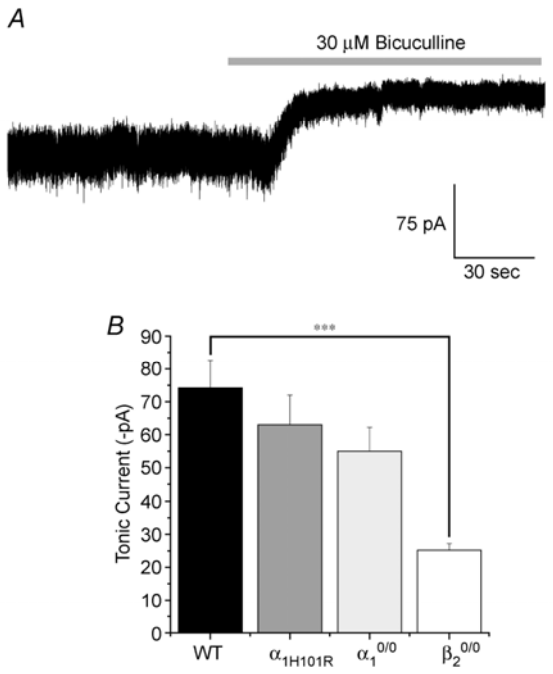


Figure 7. Deletion of the α_1 subunit does not influence the tonic current of VB neurones
A, a whole-cell recording from an exemplar $\alpha_1^{0/0}$ VB neurone. In common with WT VB neurones, the application of bicuculline reveals a large tonic conductance. Note the absence of synaptic currents under control conditions prior to bicuculline application. *B*, bar graph illustrating the magnitude of the tonic current in VB neurones of WT (black bar), α_{1H101R} (grey bar), $\alpha_1^{0/0}$ (light grey bar) and $\beta_2^{0/0}$ (open bar). Note that the tonic current recorded from $\beta_2^{0/0}$, but not α_{1H101R} or $\alpha_1^{0/0}$, VB neurones is significantly different from WT. The data for the $\beta_2^{0/0}$ neurones are reproduced from Belelli *et al.* (2005) and are shown here for comparison. Data were obtained from 7–25 recordings. Asterisks in *B* indicate statistical differences (***$P < 0.001$). Error bars indicate S.E.M.

Synaptic $GABA_A$ receptors are associated with gephyrin

Gephyrin is a marker of GABA-ergic and glycinergic postsynaptic sites in the CNS, forming clusters that are selectively colocalized with $GABA_A$ receptor subunits, as seen by immunofluorescence staining (Sassoè-Pognetto *et al.* 2000; Kralic *et al.* 2006). A systematic analysis of gephyrin clusters and their colocalization with the α_1 and α_2 subunit was therefore conducted between P10 and P30 in the VB of both WT and $\alpha_1^{0/0}$ mice. At P10, a high density of gephyrin clusters was evident, with almost 80% of them being colocalized with the α_2 subunit in both genotypes and less than 10% with the α_1 subunit in WT (Fig. 9*A*, *B* and *I*). The density of gephyrin clusters was the same in sections from mutant and WT (Fig. 9*J*). The incidence of colocalization between gephyrin clusters and the α_2 subunit decreased gradually by about 10% per day to become negligible at P20 (Fig. 9*A*–*F* and *I*, $P < 0.05$ in both genotypes, Kruskal–Wallis test), reflecting the gradual disappearance of the α_2 subunit immunoreactivity and the loss of synaptic GABA-ergic currents recorded in neurones from mutant mice (Fig. 3). In WT neurones, colocalization with the α_1 subunit was around 40% at P12–P15 and abruptly increased to almost 100% at P20 (Figs 9*I*, $P < 0.05$, Kruskal–Wallis test). We could not determine whether α_1-$GABA_A$ receptors replace α_2-$GABA_A$ receptors at the same synaptic sites, or whether the switch in subunit expression reflects the formation of novel synapses. However, the conservation of gephyrin clusters in VB neurones from $\alpha_1^{0/0}$ mice, most likely devoid of associated $GABA_A$ receptors, favours the first alternative.

Delayed alteration of gephyrin clustering in VB neurones of $\alpha_1^{0/0}$ mice

Between P10 and P30, there was a significant reduction in gephyrin cluster density with increasing age in WT and $\alpha_1^{0/0}$ animals ($P < 0.01$ in both genotypes, Kruskal–Wallis test), reflecting the growth of the VB (Fig. 9*J*). At P30, the density of gephyrin clusters in WT mice was 20% lower than at P10 (n.s.), whereas in $\alpha_1^{0/0}$ mice the reduction reached 50% ($P < 0.05$), reflecting the absence of association with the α_1 subunit. Instead, large, presumably intracellular, aggregates became apparent, as previously described in sections from adult mice (Kralic *et al.* 2006). This trend continued at later stages, with gephyrin clusters being replaced by large intracellular aggregates until about P60. At this age, no gephyrin clusters

remained in the VB of mutant mice. In view of the complete absence of mIPSCs in VB neurones from mutant mice at P23 and later, we interpret this trend as evidence for impaired gephyrin postsynaptic clustering in the absence of associated $GABA_A$ receptors.

At the subcellular level, the α_2 subunit immunoreactivity in sections from P10 and P20 mice was punctate, forming clusters extensively colocalized with gephyrin (Fig. 9*A–D*). In contrast, the α_1 subunit staining was diffuse in the neuropil, outlining the cell body of individual VB neurones (Fig. 9*G*). Although most gephyrin clusters were double-labelled for the α_1 subunit, the majority of α_1 subunit staining was not associated with gephyrin. While such a pattern is suggestive of extrasynaptic $GABA_A$ receptor labelling, it is also possible that most of the α_1 subunit immunoreactivity reflects intracellular pools of subunit protein (or assembled receptors).

Normal maturation and subcellular localization of subunits forming extrasynaptic $GABA_A$ receptors

The increase in tonic inhibition measured electrophysiologically between P10 and P20 was mirrored by a developmentally regulated expression of the α_4 subunit in the VB, as detected by immunoperoxidase staining (Fig. 10*A–D*) at these stages. No difference in the distribution, or staining intensity for the α_4 subunit was evident between WT and $\alpha_1{}^{0/0}$ mice (compare Fig. 10*A* and *B* at P10 and Fig. 10*C* and *D* at P20). Similar findings were obtained for the δ subunit (not shown), which has a very similar distribution to that of the α_4 subunit in VB. On the subcellular level, no colocalization between the α_4 subunit and gephyrin was evident in sections from WT (not shown) and mutant mice (Fig. 10*E*), whereas in the same tissue, γ_2 subunit immunofluorescence formed bright clusters colocalized with gephyrin (Fig. 10*F*). At P20, in line with the loss of postsynaptic $GABA_A$ receptors, the γ_2 subunit was almost undetectable, whereas the α_4 subunit immunoreactivity was diffuse in the neuropil (Fig. 10*G* and *H*), as reported previously for adult mice (Kralic *et al.* 2006). These results confirm the independent regulation of α subunit variants and support functional data that α_4-$GABA_A$ receptors do not contribute to synaptic transmission in VB neurones.

Long-term preservation of GABA-ergic terminals in VB of $\alpha_1{}^{0/0}$ mice

The disappearance of phasic $GABA_A$ receptor-mediated transmission and the maintenance of tonic inhibition raised the question to what extent GABA-ergic terminals are affected in the VB of $\alpha_1{}^{0/0}$ mice. Immunofluorescence staining for VGAT revealed no difference between genotypes at every age examined. At high-magnification, double labelling for VGAT and gephyrin showed that almost all gephyrin clusters were apposed to GABA-ergic terminals, which frequently appeared to form multiple postsynaptic sites (Fig. 11*A*). In sections from $\alpha_1{}^{0/0}$ mice, gephyrin intracellular aggregates were not apposed to VGAT-positive terminals, whereas the clusters remaining in the absence of α_1 subunit were still apparently postsynaptic, as shown for a section of a P30 mouse (Fig. 11*B*). A quantitative analysis confirmed these observations, showing no difference in the size of GABA-ergic terminals, as determined by cumulative distribution frequency analysis, or in their density, counted in three-dimensional volumes reconstructed from stacks of confocal images (Fig. 11*C* and *D*). The preservation of GABA-ergic terminals was also seen with immunofluorescence for GAT1 (not shown), and may account for the maintenance of tonic inhibition in the VB of mutant mice.

Discussion

Four principal findings can be derived from this study. (1) During normal development, changes in $GABA_A$ receptor subunit repertoire and expression result in functional adaptations of both phasic and tonic inhibition; however, the functional properties of synaptic immature receptors, notably the decay time constants of mIPSCs, can adapt rapidly without changes in subunit composition. (2) Tonic inhibition increases during development, independently of phasic inhibition and is maintained in adult VB neurones that lack synaptic $GABA_A$ receptors. (3) The expression profile of each $GABA_A$ receptor subtype is independent of other subunits present in VB neurones, and no functional substitution occurs between subunits contributing to synaptic and extrasynaptic receptors. And (4) the maintenance of a gephyrin postsynaptic scaffold depends on the presence of synaptic $GABA_A$ receptors; however, presynaptic terminals are retained in the absence of functional synaptic transmission.

Development of synaptic $GABA_A$ receptors

P8–9 VB GABA-ergic synapses initially contain α_2-$GABA_A$ receptors, clustered at presumptive postsynaptic sites with gephyrin. The reduced effect of a non-selective concentration of zolpidem on the decay of mIPSCs recorded from α_{2H101R} neurones confirms that these relatively slow synaptic events are mediated by α_2-$GABA_A$ receptors. Within the next 24–48 h the mIPSC decay time decreases considerably, allowing increased temporal precision, a common feature of neuronal maturation. Similar changes with development are documented for synaptic transmission mediated by ionotropic glutamate, nicotinic, glycine and $GABA_A$

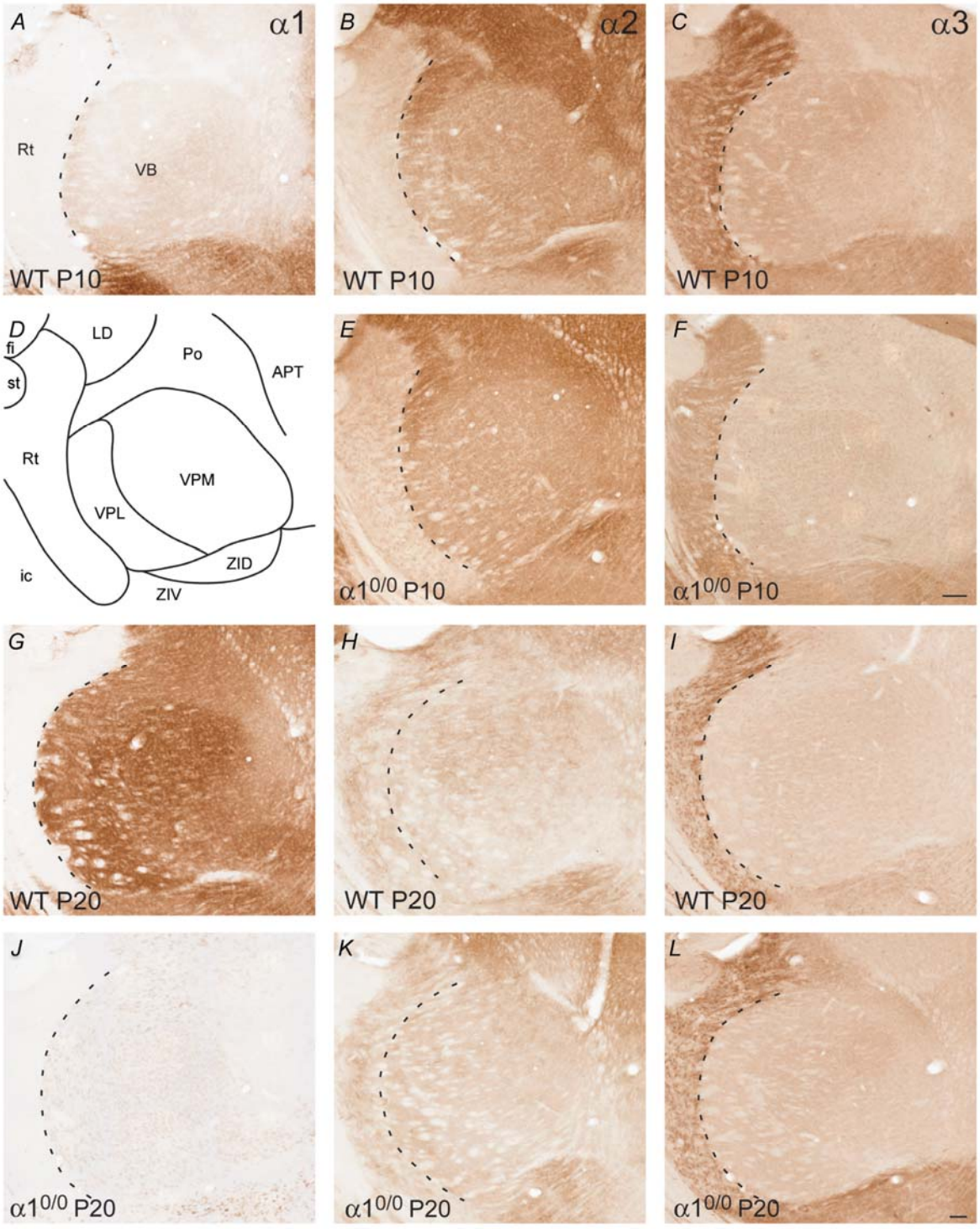


Figure 8. Comparative distribution of α_1 (*A*, *G*, *J*), α_2 (*B*, *E*, *H*, *K*) and α_3 (*C*, *F*, *I*, *L*) subunit immunoreactivity in the thalamus at P10 (*A–C*, *E*, *F*) and P20 (*G–L*) in WT (*A–C*, *G–I*) and $\alpha_1^{0/0}$ (*E*, *F*, *J–L*) mice
Parasagittal sections were processed for immunoperoxidase staining. A schematic drawing of the regions depicted is given in *D*. The α_1 subunit is conspicuously absent in VB (VPL + VPM) of P10 WT mice, but increases rapidly

receptors and changes to receptor subunit composition are often suspected (Takahashi, 2005). Inhibitory synapses of mature VB neurones express α_1-$GABA_A$ receptors (Kralic *et al.* 2006) and the appearance of this subunit has been implicated in developmental changes to synaptic transmission (Okada *et al.* 2000; Goldstein *et al.* 2002; Ortinski *et al.* 2004; Bosman *et al.* 2005). However, P10 VB neurones exhibit little α_1 subunit staining, with the majority of gephyrin clusters incorporating the α_2 subunit. In agreement, a low concentration of zolpidem, an α_1 subunit selective ligand, had little, or no effect on WT P10–11 mIPSCs, and this situation was not significantly different from that determined for neurones derived from α_{1H101R} mice. Providing further support, the mIPSCs of P10 ${\alpha_1}^{0/0}$ VB neurones were indistinguishable from WT (by P10, both strains exhibited rapidly decaying mIPSCs). Therefore, unexpectedly, the perturbation of VB mIPSC kinetics was not caused by the expression of the α_1 subunit, but reflects a rapid maturation of α_2-$GABA_A$ receptor containing synapses.

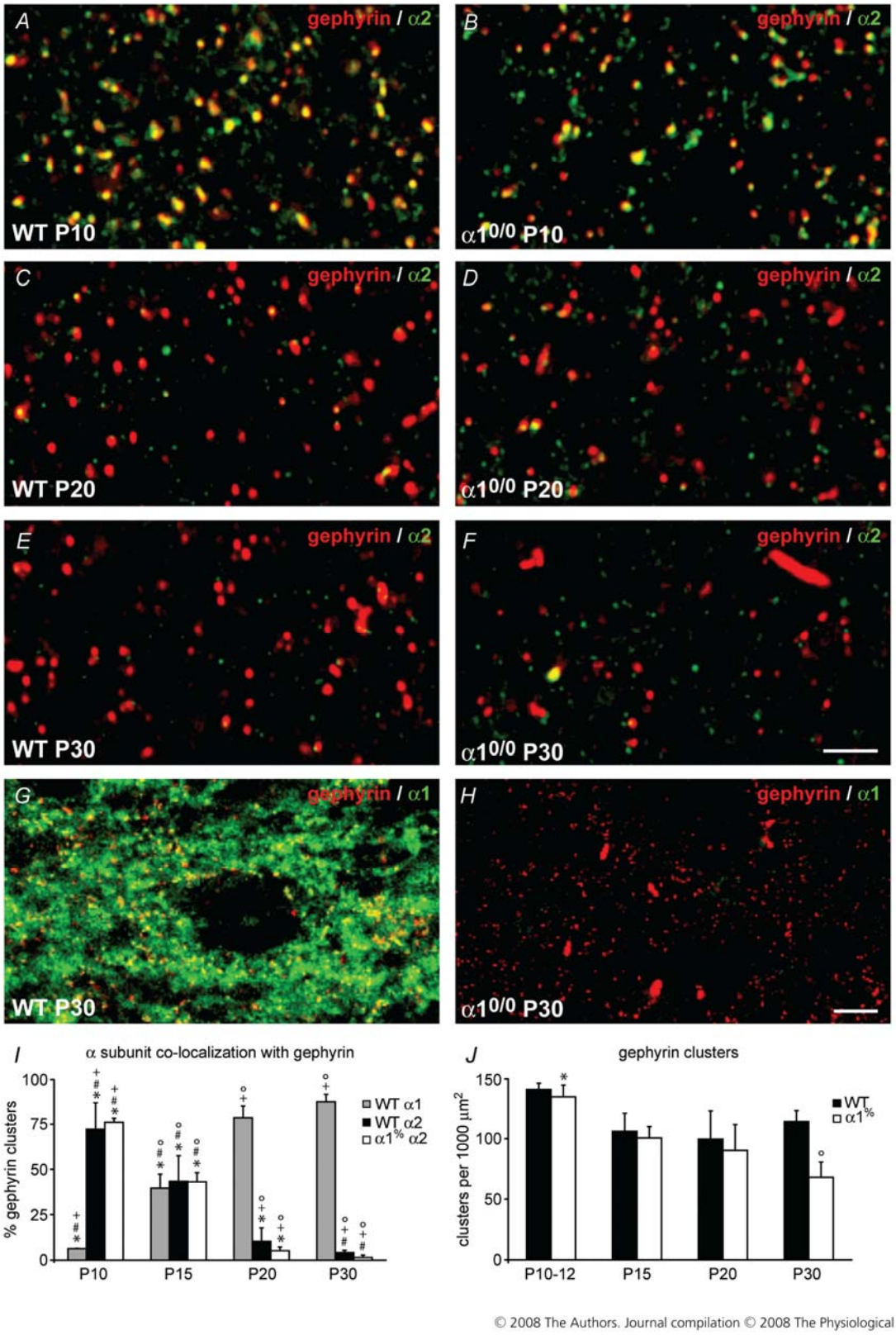
Several alternative mechanism(s) underlying these kinetic changes are conceivable: receptors containing α_4 and γ_2 subunits exhibit relatively rapid kinetics (Lagrange *et al.* 2007; Picton & Fisher, 2007). However, the α_4 subunit immunofluorescence did not colocalize with gephyrin clusters and the effects of Ro15-4513 did not support the presence of synaptic α_4-$GABA_A$ receptors in either WT, or ${\alpha_1}^{0/0}$ P10–11 neurones. In developing spinal neurones a change in neurosteroid levels influences mIPSC kinetics (Keller *et al.* 2004). Thalamic nRT neurones express neurosteroid synthesizing enzymes (Agis-Balboa *et al.* 2006), but it is not known whether neurosteroids produced in the nRT would act on the VB. Alternatively, changes to the kinetics of neurotransmitter release, expression of GABA transporters, post-translational modifications of postsynaptic proteins, or alterations of the cytoskeleton could influence mIPSC kinetics (Jones & Westbrook, 1997; Petrini *et al.* 2003; Mozrzymas, 2004; Cathala *et al.* 2005; Takahashi, 2005; Chen & Olsen, 2007). Note we also found the mIPSC decay of nRT neurones to decrease with development. In contrast to VB neurones, the subunit composition of the nRT synaptic $GABA_A$ receptors ($\alpha_3\beta_3\gamma_2$) is stable throughout development (Studer *et al.* 2006). Hence, during thalamic development, the inhibitory synaptic responses of both GABA-ergic interneurones, and thalamocortical neurones undergo kinetic changes, which are not primarily caused by a change in the $GABA_A$ receptor isoform.

During the next 10 days of development in VB neurones, the gephyrin–α_2 subunit clusters undergo considerable reorganization so that by P20, nearly all such assemblies now contain the α_1 subunit. The differential effects of zolpidem and Ro15-4513 on mIPSCs recorded from WT and α_{1H101R} VB neurones confirms the synaptic incorporation of α_1-$GABA_A$ receptors in $\geq$ P15 VB neurones. Whether the subunit switch occurs within existing synapses, or whether synapses containing α_2-$GABA_A$ receptors are replaced by novel ones incorporating the α_1 subunit is not known. The maintenance of 'orphan' gephyrin clusters for several weeks in the VB neurones of ${\alpha_1}^{0/0}$ mice favours the former scenario. The developmental loss of gephyrin–α_2 subunit clusters occurs irrespective of the α_1 subunit. In agreement, although deletion of the α_1 subunit had no effect on the synaptic responses of P8–11 VB neurones, with subsequent development the proportion of ${\alpha_1}^{0/0}$ neurones exhibiting mIPSCs decreased, until by P23–27 all neurones were 'silent'. Evidently, these neurones (post P15) cannot stray from the developmental programme and default to re-synthesize the α_2 subunit. Although increased staining of the α_4 subunit is evident in the VB neurones of ${\alpha_1}^{0/0}$ mice, this subunit does not cluster with gephyrin and the absence of mIPSCs illustrates that this extrasynaptic subunit cannot deputize for the synaptic α_1 subunit (Chandra *et al.* 2006; Kralic *et al.* 2006).

Mechanism of gephyrin clustering

Surprisingly, the gephyrin clusters of VB ${\alpha_1}^{0/0}$ mice remained for an extended time at presumptive postsynaptic sites devoid of functional $GABA_A$ receptors. In juvenile mice conditional knockout of the γ_2 subunit in cerebral cortex and hippocampus caused gephyrin clusters to disappear within a few days (Schweizer *et al.* 2003). Although the γ_2 subunit was not targeted in ${\alpha_1}^{0/0}$ mice, it also loses its' postsynaptic localization, suggesting cell-specific differences in the stability of gephyrin clusters. Whether these differences reflect the molecular heterogeneity of gephyrin, or other components of GABA-ergic synapses is not established. In this regard, collybistin-null mice exhibit striking regional differences in the loss of postsynaptic gephyrin/$GABA_A$ receptor

thereafter. In contrast, the α_2 subunit staining is moderate at P10 and decreases to background levels by P20 in both WT and ${\alpha_1}^{0/0}$ mice. Finally, the α_3 subunit immunoreactivity, which is intense in Rt in both genotypes, is not detectable in VB at either age. Abbreviations: APT, anterior pretectal nucleus; fi, fimbria of the hippocampus; ic, internal capsule; LD, laterodorsal thalamic nucleus; Po, posterior thalamic nuclear group; Rt, reticular thalamic nucleus; st, stria terminalis; VPL; ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus; ZID, zona incerta, dorsal part; ZIV, zona incerta, ventral part. Scale bars, 100 μm (scale bar in *F* applies to *A–F*, scale bar in *L* applies to *G–L*).



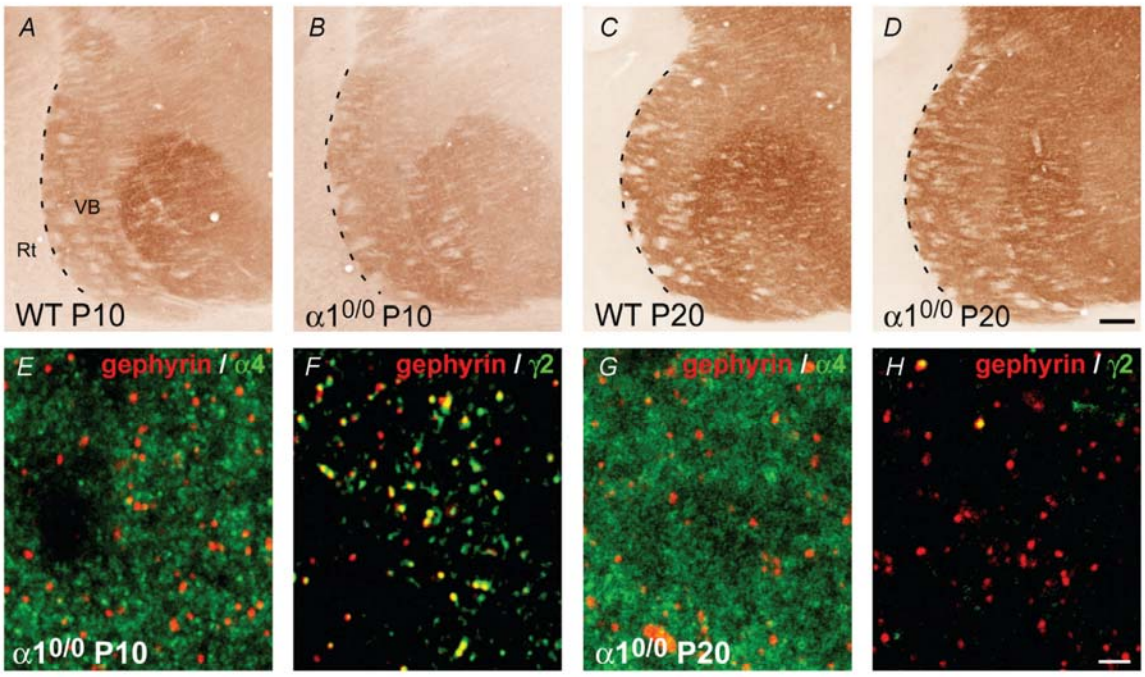


Figure 10. Comparative distribution of the α_4 subunit immunoreactivity in VB of WT and $\alpha_1^{0/0}$ mice at P10 and P20, as illustrated by immunoperoxidase staining
A, B, A moderate staining for the α_4 subunit selectively present in VB, but not in the nRT, is already evident at P10, with a similar distribution in both genotypes. C, D, The staining intensity increases until P20, without revealing differences between WT and $\alpha_1^{0/0}$ mice at this stage. *E–H*, double immunofluorescence for gephyrin (red) and the α_4 (green; *E* and *G*) or γ_2 (green; *F* and *H*) subunit at P10 and P20 in $\alpha_1^{0/0}$ mice; the extrasynaptic localization of the α_4 subunit is inferred from its lack of colocalization with gephyrin, despite the absence of the α_1 subunit at either stage. Note that the γ_2 subunit staining intensity decreases dramatically between P10 and P20, reflecting the loss of mIPSCs recorded electrophysiologically. Scale bars: *D* (applies for *A–D*), 100 μm; *H* (applies for *E–H*), 2 μm.

clusters in the absence of this GDP/GTP-exchange factor, known to interact with gephyrin (Papadopoulos *et al.* 2007).

Development of GABA-ergic terminals

Although the mean absolute number of GABA-ergic synapses per VB neurone could not be quantified morphologically (using VGAT staining), an increase in synaptic coverage per relay neurone is likely, given the increased frequency of mIPSCs during development and the similar density of gephyrin clusters seen in WT mice, despite the considerable growth of the neuropil in VB between birth and P30. Analysis of GABA-ergic terminals with VGAT revealed no detectable morphological alteration in VB neurones of $\alpha_1^{0/0}$ mice,

Figure 9. Differential alterations in postsynaptic clustering of the α_2 (green; *A–F*) and α_1 subunit (green; *G–H*) and gephyrin (red, *A–H*) during development of WT (left column) and $\alpha_1^{0/0}$ (right column) mice, visualized by immunofluorescence staining and confocal laser scanning microscopy
At P10 (*A* and *B*) most gephyrin clusters are colocalized with the α_2 subunit in both genotypes. This fraction gradually decreases between P10 and P30 (*A–F*, quantified in *I*), due to the loss of α_2 subunit staining, which is replaced by the α_1 subunit in WT (*G*, quantified in *I*), but not in mutants (*H*). As a result, gephyrin clusters in $\alpha_1^{0/0}$ mice remain isolated, and at P30, gephyrin forms large aggregates (*F* and *H*). *I*, quantification of colocalization patterns between gephyrin and the α_1 or α_2 subunit in WT and $\alpha_1^{0/0}$ mice. Pair-wise significant differences between age groups are indicated by symbols (*$P < 0.05$ compared to P30; #$P < 0.05$ compared to P20; +$P < 0.05$ compared to P15; °$P < 0.05$ compared to P10). *J*, quantification of gephyrin cluster density in WT and $\alpha_1^{0/0}$ mice. No change is evident in WT, whereas a 30% decrease occurs in mutants between P10 and P30; the same symbols are used as in *I*. Scale bar in *F*, 3 μm (applies to *A–F*), in H, 10 μm (applies to *G* and *H*).

contrasting with the changes observed in the cerebellum of $\alpha_1^{0/0}$ mice and in the nRT of $\alpha_3^{0/0}$ mice (Kralic *et al.* 2006; Studer *et al.* 2006). Since VGAT is the vesicular transporter for GABA (and glycine), the storage of GABA in presynaptic vesicles remains operant and probably sustains the tonic GABA-ergic inhibition in VB neurones. The long-term maintenance of postsynaptic sites is therefore not essential for GABA-ergic terminals. In the cerebellum, immunoelectron microscopy reveals that such 'orphan' terminals make aberrant synapses with the spines of Purkinje cells (Fritschy *et al.* 2006). Whether they find a novel postsynaptic partner in VB neurones is not known.

Development of extrasynaptic $GABA_A$ receptors

The tonic current increased considerably (~4-fold) during postnatal weeks 3–4. Therefore, considering the total charge transfer in developing VB neurones the emphasis is switched from synaptic transmission, which is temporally and spatially restricted, to asynchronous activation of extrasynaptic receptors. Experiments with THIP and our immunohistochemical data suggest an increased $\alpha_4\delta$-$GABA_A$ receptor expression primarily accounts for the increase in tonic inhibition.

Although a large proportion of the α_1 subunit staining in WT mice is not associated with gephyrin, we found no evidence for a contribution of α_1-$GABA_A$ receptors to tonic inhibition. Therefore, this immunohistochemical signal may represent intracellular α_1 subunit protein. The predominant contribution of α_4-$GABA_A$ receptors to tonic inhibition is also supported by the fact that deletion of this subunit selectively abolished the VB tonic conductance, with no effect on IPSCs (Chandra *et al.* 2006). Hence, the α_1 subunit cannot deputize for the extrasynaptic α_4 subunit, nor can α_4 replace the synaptic α_1 subunit. This

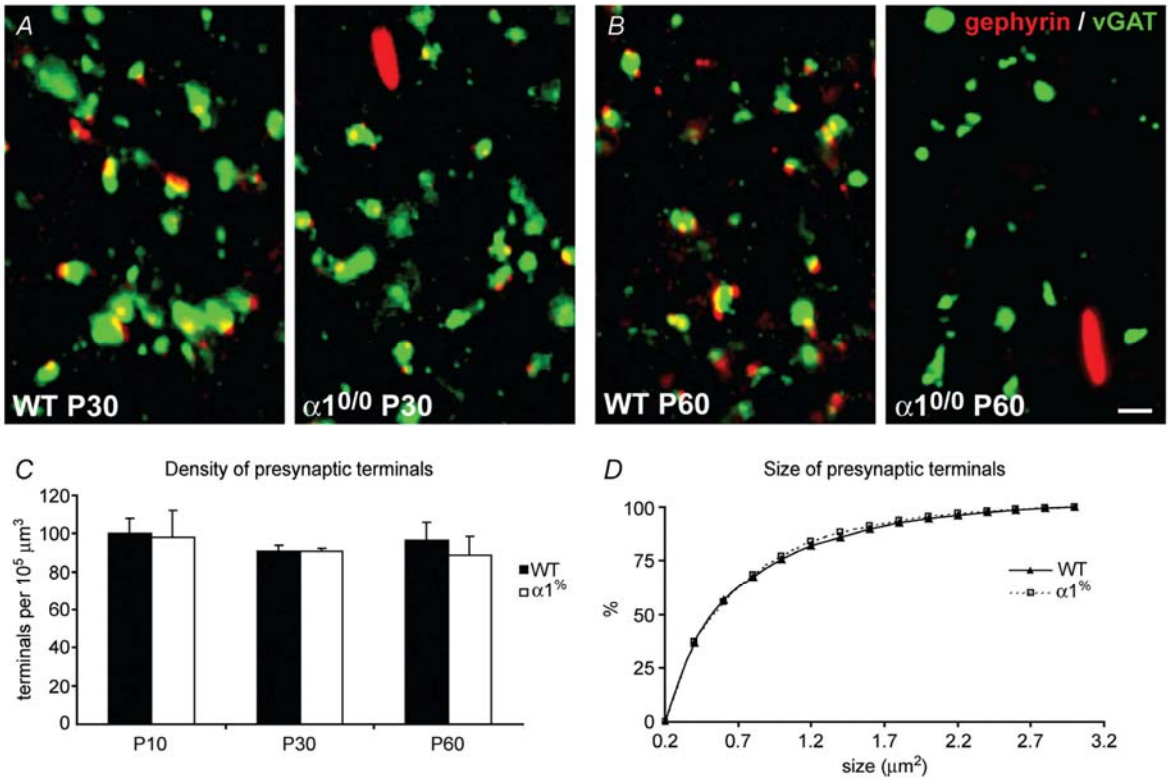


Figure 11. GABA-ergic terminals remain unaffected in the VB of $\alpha_1^{0/0}$ mice during development, as seen by double immunofluorescence staining (*A*, *B*) of vGAT (green) and gephyrin (red)
A, at P30, the close apposition of both markers confirms the postsynaptic localization of gephyrin clusters in both genotypes, whereas large aggregates are not associated with gephyrin. *B*, at P60, most gephyrin clusters have disappeared and are replaced by aggregates. *C*, the density of vGAT positive terminals formed at P10 remains constant until adulthood and does not differ between WT and $\alpha_1^{0/0}$ mice. *D*, likewise, their size, as determined by cumulative distribution analysis, is comparable in adult and WT $\alpha_1^{0/0}$ mice, despite the loss of postsynaptic proteins occurring in the mutants. Scale bar in *B*, 2 μm (applies to *A* and *B*).

specificity appears host specific as receptors composed of α_1 and δ subunits are readily expressed in cell lines and hippocampal interneurones (Wohlfarth *et al.* 2002; Glykys *et al.* 2007).

In contrast to the target specificity of the α subunit, in $\geq$ P15 VB neurones the β_2 subunit is expressed both synaptically and extrasynaptically (Belelli *et al.* 2005) and appears synchronously ($\sim$P15) in these distinct receptor populations, suggesting a coordinated synthesis. Similarly, we find extrasynaptic receptors of dentate gyrus granule cells (DGGCs) to incorporate the β_2 subunit (Herd *et al.* 2008). Therefore, $\alpha_4\beta_2\delta$ receptors are expressed in two distinct neuronal populations.

In conclusion, during ontogeny, thalamic relay neurones undergo a considerable reorganization of inhibitory synapses, coincident with the establishment of extrasynaptic $GABA_A$ receptors. The time course of synaptic inhibition can influence neuronal signal integration and consequently network activity (Jefferys *et al.* 1996). Therefore, the developmental changes to the duration of synaptic inhibition may have an impact on thalamo-cortical oscillations. In rat (P14–21) VB neurones activation of extrasynaptic $GABA_A$ receptors causes a hyperpolarization that leads to a 'burst firing' mode (Cope *et al.* 2005). However, in many developing neurones GABA produces a depolarization, due to an immature Cl^- gradient. Therefore it will be important to investigate the nature of the GABA response during the period associated with the development of the tonic conductance. Future studies on the impact of development to thalamic network activity should permit a better understanding of how synaptic and extrasynaptic $GABA_A$ receptors act in concert to accommodate the challenges of neuronal maturation.

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